PLANT PHYSIOLOGY

VOLUME 33 JULY, 1958 NUMBER 4

MANGANESE REQUIREMENT WITH RESPECT TO GROWTH, HILL REACTION AND PHOTOSYNTHESIS[1]

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The necessity of manganese for the growth of both autotrophic and heterotrophic plants was reported first by Bertrand (5, 6) more than 50 years ago, and the essentiality of manganese for the growth of Chlorella was shown originally by Hopkins (20) of Cornell University in 1930.

The relation of manganese to photosynthesis was suspected by McHargue (24) and Bishop (7). However, it was not until 1937 when Pirson (25) showed that the photosynthesis of manganese-deficient Chlorella cells was partially inhibited and that the inhibition could be relieved instantaneously by the addition of manganese to the medium. Emerson and Lewis (12, 13) observed that the absence of micronutrient elements in the culture medium caused a decrease in the quantum yield of photosynthesis, and that the efficiency of photosynthesis was improved more with the addition of manganese than with the addition of any other micronutrient element. The literature on this subject has been reviewed by Pirson (26). More recently, there has been the work of Kessler (21), Habermann (18), Pirson and Bergmann (27) and Reisner and Thompson (31, 32, 33).

This paper reports a study of the growth, Hill reaction, and photosynthesis of *Chlorella pyrenoidosa* cultured autotrophically and heterotrophically at various levels of managanese, and similar studies with autotrophically cultured *Nostoc muscorum, Scenedesmus quadricauda, Porphyridium cruentum* and *Lemna minor*. Measurements pertaining to the chlorophyll-manganese ratio and the relation of manganese both to the chlorophyll content and to the destruction of chlorophyll by light are also included. Preliminary results have already been published (14), and some of the present results were reported at the 1956 meeting of the American Institute of Biological Sciences (15).

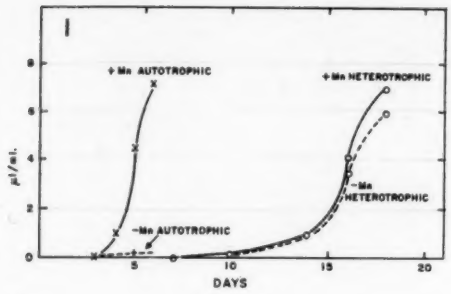
METHODS

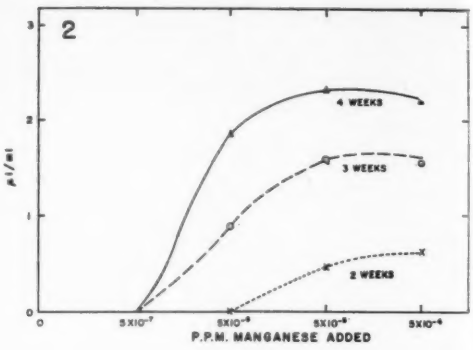
Chlorella pyrenoidosa (Emerson strain) was obtained from Dr. Jack Myers of the University of Texas. This alga was cultured in Warburg and Burk medium (34) supplemented with 1 ppm Fe as $FeSO_4 \cdot 7\,H_2O$, 0.02 ppm Cu as $CuSO_4 \cdot 5\,H_2O$, 0.5 ppm B as H_3BO_3, 0.05 ppm Zn as $ZnSO_4 \cdot 7\,H_2O$, and 0.01 ppm Mo as $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$. The manganese was added separately as $MnSO_4 \cdot 7\,H_2O$ from a dilution series. Fisher Certified and Baker Analyzed reagents supplied the macronutrients, and Matthey Specpure compounds furnished the micronutrients. *Scenedesmus quadricauda* and *Porphyridium cruentum* were obtained from the University of Indiana Algae Culture Collection. The former was cultured on a modified Chicago medium containing 1 liter H_2O, 500 mg $MgSO_4 \cdot 7\,H_2O$, 250 mg KNO_3, 250 mg K_2HPO_4, 200 mg NaCl, 27 mg Specpure $CaCl_2$, 1.88 millimoles NaOH prepared from C. P. Baker's Analyzed metallic sodium, and supplemented with iron and trace elements as for Chlorella. *Porphyridium cruentum* was cultured in a medium formulated as follows: 1 liter H_2O, 2.5 g NH_4NO_3, 3.75 g $Mg(NO_3)_2 \cdot 6\,H_2O$, 6.7 g NaCl, 625 mg KCl, 300 mg $CaCl_2 \cdot 2\,H_2O$, 24 mg K_2SO_4, 25 mg KH_2PO_4, 649 mg $NaHCO_3$, and supplemented with iron and trace elements as for Chlorella, with molybdenum increased from 0.01 ppm to 0.1 ppm. *Nostoc muscorum*, obtained from Dr. G. C. Gerloff, University of Wisconsin, was cultured on a modified Chu medium containing 1 liter H_2O, 125 mg $MgSO_4 \cdot 7\,H_2O$, 250 mg KNO_3, 30 mg K_2HPO_4, 27 mg Specpure $CaCl_2$, 3.76 millimoles NaOH specially prepared as described above, and supplemented with iron and trace elements as for Porphyridium. *Lemna minor*, from the Carolina Biological Supply Co. was cultured in Hoagland's medium (17).

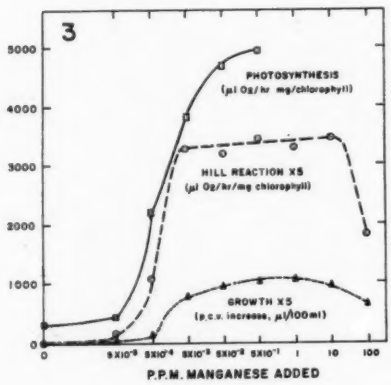
The glucose for heterotrophic growth was dissolved double-strength in water, autoclaved, and when cool added to autoclaved double-strength medium. The final concentration in the medium was 1%. For preparing the low-manganese medium, glucose was purified by passing a 10% solution in redistilled water successively through two 1.1×25 cm Pyrex columns containing 50-100 mesh Dowex 50—X2 cation exchange resin which was operated in the hydrogen cycle.

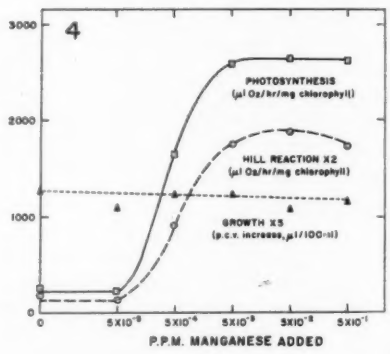
The water was redistilled in a Pyrex-glass distillation apparatus and the glassware was supercleaned by the method of Waring and Werkman (35). The culture flasks were 300-ml Pyrex flasks with side arms, containing 100 ml of medium, plugged with cotton and covered with rubber caps. The cultures

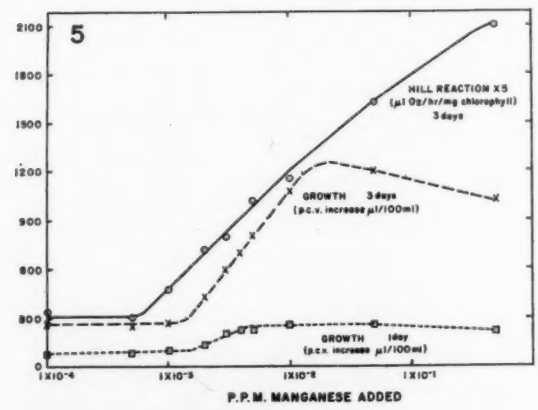
[1] Received November 18, 1957.











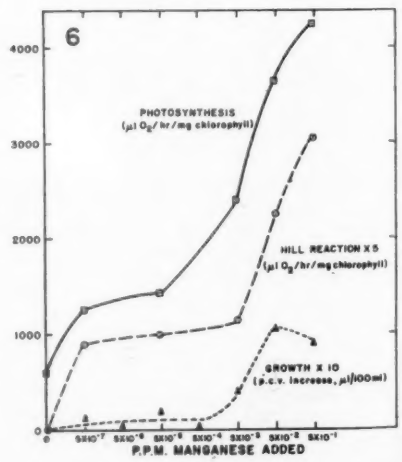


FIG. 1. Autotrophic and heterotrophic growth of *Chlorella pyrenoidosa* without added manganese and with 0.5 ppm added manganese in the culture medium.

FIG. 2. Heterotrophic growth of *Chlorella pyrenoidosa* at very low concentrations of added manganese.

were bubbled with 5 % CO_2 in air at the rate of about 35 ml/min, agitated continuously on a shaker in a room maintained at 25° C, and illuminated with approximately 1000 ft-c of daylight fluorescent light.

The heterotrophic Chlorella cultures were started with one loopful of an autotrophic bacteria-free stock culture which contained 0.5 ppm manganese. The manganese series of autotrophic cultures were begun from a minus-manganese heterotrophic culture that had been washed to remove the glucose. Fifty to 100 μl of washed cells per 100 ml of culture were used.

The procedure for the determination of chlorophyll in Chlorella and Scenedesmus was based on the work of MacKinney (23), using methanol extraction. Chlorophyll determinations for Nostoc were obtained by Arnon's method for chloroplasts (3) using acetone since methanol did not satisfactorily remove the chlorophyll. Standard procedures were used for the isolation of chloroplasts from *Lemna minor* (9) and for the determination of chlorophyll in these chloroplasts (3).

Manometric measurements of photosynthesis and of the Hill reaction have been described by Brown (8), and Clendenning and Ehrmantraut (10), respectively. For measurements of photosynthesis, manganese-free 0.1 M sodium carbonate-bicarbonate buffer at pH 9.4 to 9.5 was prepared from the specially synthesized NaOH which had been used in the culture media. All of the results reported are based on the direct method of Warburg, although some photosynthetic measurements have been obtained with the indirect method.

Results and Discussion

Figure 1 compares the growth rates of Chlorella grown autotrophically and heterotrophically without added manganese and with 0.5 ppm added manganese. Heterotrophic growth showed a lag period of about 10 days. The most rapid growth occurred in plus-manganese cultures grown autotrophically. Minus-manganese autotrophic cultures did not produce visible growth. Pirson and Bergmann (27) and Bergmann (4), reported a reduction in growth of the minus-manganese autotrophic cultures to about 50 % of that of non-deficient cultures. There was no measurable autotrophic growth of the manganese-free cultures grown by Reisner and Thompson (33) in a medium prepared with purified nutrients. As early as 1930 Hopkins (20) reported that he was able to reduce autotrophic growth 10- to 600-fold in culture solutions from which the contaminating manganese had been removed by adsorption on calcium phosphate.

Growth in the minus-manganese heterotrophic cultures was 50 to 70 % of that in plus-manganese heterotrophic control cultures. The manganese contamination in the minus-manganese medium was further reduced by raising a heterotrophic crop of Chlorella in it. Heterotrophic growth of the second crop in this used medium was 34 % of the growth in the same used medium to which 0.5 ppm manganese had been added.

The complete elimination of heterotrophic growth was achieved by reducing the concentration of the nutrients 100-fold for the macronutrients and 10-fold for the iron and micronutrients, thus minimizing the contaminating manganese. There was no growth in this medium with 5×10^{-7} ppm Mn (10^{-11} M) added, but growth did occur with 5×10^{-6} ppm Mn (10^{-10} M) or more added (fig 2). The culture with 5×10^{-6} ppm Mn was appreciably slower in getting started than the cultures with higher concentrations of manganese. Pirson and Bergmann (27) obtained no reduction in growth in minus-manganese heterotrophic cultures compared with cultures containing 0.056 ppm Mn. However, Reisner and Thompson (33) were able to show reduction in the growth of heterotrophic manganese-deficient Chlorella cultures down to 75 % of the manganese-sufficient cultures.

Growth, Hill reaction and photosynthesis of autotrophic Chlorella cultures containing various concentrations of manganese were found to closely parallel each other (fig 3). Those with zero or 5×10^{-5} ppm added manganese usually showed no growth, no Hill reaction activity, and low photosynthesis. As the manganese was increased to 5×10^{-4} ppm there was very slow growth, and a small corresponding increase in both Hill reaction and photosynthesis. Most of the increase consistently occurred between 5×10^{-4} ppm and 5×10^{-3} ppm manganese, and fairly uniform values resulted at the higher manganese concentration levels. Furthermore, there was consistently somewhat less growth at 5×10^{-1} ppm than at 5×10^{-2} ppm, and growth declined progressively at still higher levels of manganese. Hill reaction values for Chlorella cells grown with 100 ppm manganese showed a marked decline from the values for Chlorella cells grown with 10 ppm manganese.

In order to show the low photosynthetic rate, 6 % of normal, at the lowest manganese concentrations in

Fig. 3. Growth, Hill reaction and photosynthesis of *Chlorella pyrenoidosa* cultured 1 day autotrophically at various concentrations of added manganese.

Fig. 4. Growth, Hill reaction and photosynthesis of *Chlorella pyrenoidosa* cultured 18 days heterotrophically at various concentrations of added manganese.

Fig. 5. Growth and Hill reaction measurements of *Chlorella pyrenoidosa* cultured autotrophically at close step concentrations of added manganese in the region of pronounced increase. Plotted on semi-log scale, the point between 1×10^{-4} and 1×10^{-3} is 5×10^{-4}. The points between 1×10^{-3} and 1×10^{-2} are 2, 3, 4 and 5×10^{-3}. The two highest points are 5×10^{-2} and 5×10^{-1}.

Fig. 6. Growth, Hill reaction and photosynthesis of *Scenedesmus quadricauda* cultured 1 day autotrophically at various concentrations of added manganese.

figure 3, it was necessary to use specially purified manganese-free sodium carbonate-bicarbonate buffer in the Warburg vessels.

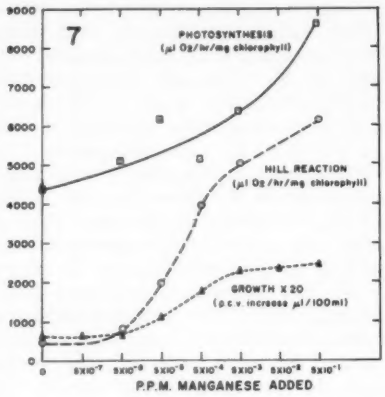
Figure 4 shows that although heterotrophic growth of Chlorella was the same at all of the levels of manganese concentration tried, there was Hill reaction activity and capacity for photosynthesis only if manganese exceeded a minimum value in the cultures. It has been shown repeatedly that "zero-manganese" heterotrophic cultures have no Hill reaction activity and no capacity for photosynthesis. The pronounced increase in Hill reaction values and photosynthesis occurred between 5×10^{-5} ppm and 5×10^{-3} ppm manganese.

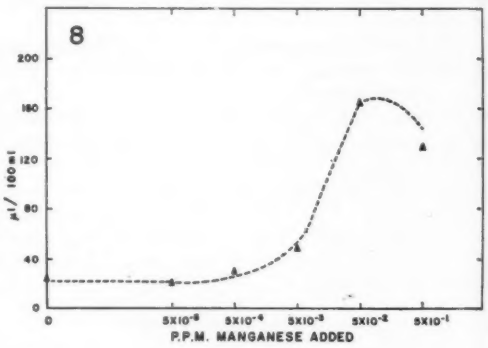
Figure 5 gives the results of an experiment which was designed to yield more detailed information on the portion of the manganese concentration growth curve where autotrophic growth and Hill reaction values showed a pronounced increase. Both one-day- and three-day-growth curves and Hill reaction measurements on the three-day cultures are shown. Autotrophic growth began its pronounced increase at a point slightly above 1×10^{-3} ppm manganese. There was a straight line relationship of Hill reaction values for cultures grown with manganese concentrations between 5×10^{-4} ppm and 1×10^{-2} ppm.

The very small but definite manganese requirement for heterotrophic growth of Chlorella suggests that it is a necessary co-factor of an enzymatic system. A requirement for manganese has been shown by Anderson and Evans (2) for *iso*-citric dehydrogenase and the "malic enzyme," and by Kornberg et al (22) for oxalosuccinic decarboxylase.

The 1000-fold larger manganese requirement for autotrophic growth, and the parallel responses of autotrophic growth, Hill reaction and photosynthesis with respect to manganese content, strongly indicate that manganese is an essential component of that part of the photosynthetic apparatus which is common to these three processes. This conclusion is compatible with the suggestion of Pirson et al (28) and with the further evidence of Kessler (21) that manganese is involved in the photosynthetic liberation of oxygen.

Scenedesmus quadricauda, *Nostoc muscorum*, *Porphyridium cruentum*, and *Lemna minor* were made manganese deficient by repeated autotrophic sub-culturing until zero Hill reaction activity was attained. The response to the addition of various concentrations of manganese was observed to be generally the same as that shown by Chlorella (figs 6 to 9). Scenedesmus, Porphyridium, and Lemna, however, required higher levels of manganese, whereas Nostoc responded to lower levels of manganese than Chlorella. There was a small but significant increase in growth and Hill reaction of Nostoc at 5×10^{-5} ppm manganese, a concentration which has consistently been insufficient to cause Chlorella to respond. The possibility that this variation shown by Nostoc may have been due to the medium was ruled out by an experiment in which Chlorella was grown in modi-





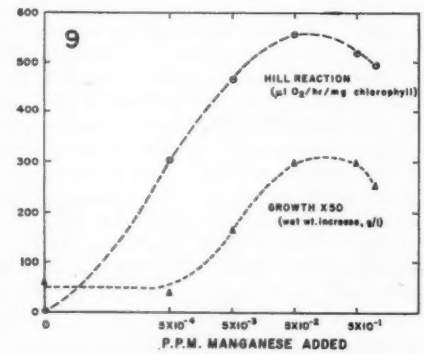


FIG. 7. Growth, Hill reaction and photosynthesis of *Nostoc muscorum* cultured 1 day autotrophically at various concentrations of added manganese.

FIG. 8. Growth of *Porphyridium cruentum* cultured 7 days autotrophically at various concentrations of added manganese.

FIG. 9. Growth and Hill reaction of *Lemna minor* cultured 4 days autotrophically at various concentrations of added manganese.

TABLE I

CHLOROPHYLL CONTENT OF ALGAE CULTURED AT DIFFERENT LEVELS OF MANGANESE

ORGANISM	TYPE OF CULTURE	CHLOROPHYLL CONTENT AT INDICATED Mn CONCENTRATION					
		ZERO	5×10^{-5} PPM	5×10^{-4} PPM	5×10^{-3} PPM	5×10^{-2} PPM	5×10^{-1} PPM
		mg/ml p.c.	*mg/ml p.c.*	*mg/ml p.c.*	*mg/ml p.c.*	*mg/ml p.c.*	*mg/ml p.c.*
Chlorella	Autotrophic	6.00	5.30	6.20	5.30	5.25	4.80
Chlorella	Heterotrophic	6.25	6.20	4.50	4.50	4.65	5.20
Scenedesmus	Autotrophic	3.52 *	3.90 *	3.62 *	4.60	2.67	2.86
Nostoc	Autotrophic	1.22	1.40	1.44	1.75	1.90	1.68

* Incomplete extraction.

fied Chu medium. It was found that the same concentration of manganese was required to support autotrophic growth, Hill reaction and photosynthesis as was required in the modified Warburg and Burk medium.

Measurements of Hill reaction and photosynthesis on *Porphyridium cruentum* were not attempted. In the case of *Lemna minor*, the Hill reaction was determined on isolated chloroplasts.

These results indicate that the photosynthetic role of manganese is quite general, extending to at least several species of algae with different pigment systems and to higher plants.

Table I presents the chlorophyll content of one-day-old autotrophic cultures of three different algae, and that of an 18-day-old heterotrophic culture of Chlorella, for various concentrations of manganese in the medium.

Prolonged continuous illumination of manganese-deficient autotrophic cultures of Chlorella caused a bleaching effect and resulted in marked chlorosis. Two Chlorella cultures, one with 0.5 ppm Mn added and another with no Mn added, which were exposed to about 1000 ft-c of light autotrophically for three days, were found to contain 7.5 mg and 0.9 mg chlorophyll per ml of packed cells, respectively. When the experiment was repeated, values of 11.3 mg and 2.3 mg chlorophyll per ml of packed cells were obtained.

There are conflicting reports on the chlorophyll content of manganese-deficient plants. Bishop (7) reported that manganese deficiency interfered with the synthesis of chlorophyll thereby affecting carbon assimilation. Portsmouth (29) found that manganese-treated potato leaves became very much greener and healthier looking than the manganese-deficient controls. However, Pirson et al (28) as well as others generally agree that manganese deficiency does not directly produce chlorosis. Rao and Lal (30) studied the effect of manganese deficiency on barley plants and reported that the chlorophyll content of the manganese-deficient plants was more than double that of the controls.

The pronounced protective action of manganese against photobleaching of the chlorophyll in Chlorella may also indicate that it is a component of the oxygen disposal system. Photobleaching can result from the oxidizing action of peroxides which are formed by the reduction of oxygen. Thus, the efficient removal of oxygen would decrease photobleaching.

Radioactive manganese was used to determine the manganese content of one-day-old autotrophic Chlorella cells which were cultured in a medium containing the concentration of manganese (5.0×10^{-3} ppm) just sufficient for maximum growth. The manganese was found to be 114 times more concentrated in the cells than in the medium. Hence, every μl packed cells contained 1.0×10^{-11} moles of manganese. Since there were 50×10^6 cells per μl packed cell volume each cell had an average of 120,000 atoms of manganese. Identically grown cells had a chlorophyll content of 5.3 mg per ml packed cell volume (table I) or an average of 7×10^7 chlorophyll molecules per cell. Thus, there is a ratio of about 600 chlorophyll molecules per atom of manganese at the concentration where manganese ceased to be a rate limiting factor. It is interesting to compare this ratio with the calculations of Emerson and Arnold (11) who measured the maximum oxygen yield per flash of light. They found roughly one mole of oxygen for every 2500 moles of chlorophyll for a Chlorella culture comparable to ours. There are four electrons required to form one molecule of oxygen from water, so there has been one electron transferred for every 625 chlorophyll molecules. The general agreement with the manganese-chlorophyll ratio suggests that, in some cases, the limiting factor for photochemical oxygen evolution may be the manganese component. Clendenning and Ehrmantraut (10) reported that there was a rate-limiting catalyst which controlled maxi-

TABLE II

CELL NUMBERS, CELL VOLUMES, AND CELL DIAMETERS OF HETEROTROPHICALLY GROWN NORMAL AND MANGANESE DEFICIENT CHLORELLA

	NORMAL + Mn	ZERO Mn
Average cell number per μl p.c.	43.5×10^6 cells	21.4×10^6 cells
Average cell volume	23.0 μ^3	46.7 μ^3
Average cell diameter	3.5 μ	4.5 μ

TABLE III

RECOVERY OF HILL REACTION IN MANGANESE DEFICIENT CHLORELLA PYRENOIDOSA, UPON INCUBATION AT 25° C IN THE LIGHT WITH ADDED MANGANESE

EXPERIMENT NUMBER	SUBSTRATE	INCUBATION TIME HR	MANGANESE ADDITION PPM	HILL REACTION	
				CONTROL ML O_2/HR	Mn ADDED ML O_2/HR
43 *	PO_4 buffer	0.5	0.5, $MnSO_4$	2.4	30.9
	"	1	" "	2.4	41.7
105	"	16	0.6, $Mn^{+2}SO_4$	12.4	25.0
	"	16	0.6 $KMn^{+7}O_4$	12.4	24.8
152	W-B medium	1	0.017, $MnSO_4$	11.6	32.0
	PO_4 buffer	1	" "	19.6	28.2
	TRIS buffer	1	" "	17.2	29.2

* Extremely deficient culture.

mum oxygen evolution from intact cells of *Chlorella pyrenoidosa* during both photosynthesis and quinone Hill reactions.

There was a marked difference in size, growth habit, and morphology between normal and manganese-deficient heterotrophic Chlorella cells. Normal heterotrophic cells were slightly larger than normal autotrophic cells, and deficient heterotrophic cells had twice the volume of normal heterotrophic cells (table II). Deficient cells had a pronounced tendency to clump and the chloroplasts were poorly defined. Alberts-Dietert (1) also observed the relation of Chlorella cell size to manganese supply.

A good Hill reaction rate was readily obtained by adding manganese to deficient cells. The deficient cells were suspended in the culture medium containing the manganese compound and were incubated in the light (approx. 1000 ft-c) at room temperature for one hour. Some of the results are reported in table III. These results indicate that there were no differences in recovery due to using manganese of different valence states. The importance of incubation time and substrate are also shown. The comparatively rapid response of photosynthesis and Hill reaction when manganese was added to a deficient culture indicates direct participation rather than a secondary action.

In order to rule out the possibility that the manganese effect on the Hill reaction could be unique to the presence of quinone as a Hill reaction oxidant, other oxidants were tested. Hill's reagent (19), and benzaldehyde (16) were used in comparison with quinone. Similar results were found in all cases, with both manganese-deficient and normal Chlorella. Activity differences between "minus" Mn and "plus" Mn cells with the different oxidants were as follows: benzaldehyde > quinone > Hill's reagent.

SUMMARY

Chlorella pyrenoidosa required a minimum of 10^{-7} ppm manganese in the culture medium for heterotrophic growth and a 1000-fold larger amount for autotrophic growth. Autotrophic growth, Hill reaction, and photosynthesis responded equally when increments of manganese were added to manganese-deficient cultures. Similar results were obtained with *Scenedesmus quadricauda, Nostoc muscorum, Porphyridium cruentum,* and *Lemna minor.* The chlorophyll content of one-day-old autotrophic cultures of Chlorella and Scenedesmus was not affected adversely by manganese deficiency, but after being illuminated three days, manganese deficient cultures of Chlorella had much less chlorophyll than similarly treated non-deficient cultures. When manganese was added to deficient cultures, their capacities for photosynthesis and Hill reaction were quickly restored. Chlorella cultured in a medium containing manganese just sufficient for full photosynthesis and Hill reaction had a manganese-chlorophyll molar ratio of 1 to 600. The results indicate that a very small amount of manganese suffices as an essential enzymatic co-factor for heterotrophic growth, and that a much larger amount forms an essential part of the photosynthetic oxygen-evolving apparatus as previously suggested by others.

NaOH was prepared from C. P. Baker's Analyzed reagent grade sodium by J. Eichel, Chemist. Modifications in chlorophyll determinations for Nostoc and Scenedesmus were developed by Miss Joan Groulx. Cell counts were done by Miss Grace Norris, and the drawings were prepared by Richard Miller.

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LIGHT-DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. I. STIMULATION FOLLOWING QUINONE REDUCTION[1,2]

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The term "Hill reaction" has been used to describe any light-dependent chloroplast reaction in which a suitable oxidant reacts with the reduced product of the splitting of water and in which oxygen is evolved from the oxidized product. Mehler (7) first showed that oxygen could function as a Hill reagent, i.e., as an oxidant which is effective in promoting photochemical oxygen production by chloroplast preparations. In Mehler's experiments, chloroplast preparations to which excess catalase and ethanol had been added as a sink for H_2O_2 (6) reduced molecular oxygen to hydrogen peroxide in the light while they simultaneously evolved oxygen derived from water. The peroxide was removed by a coupled oxidation of ethanol to acetaldehyde. In this reaction, then, there was for each mole of oxygen reduced an accompanying production of 0.5 mole yielding a net uptake of 0.5 mole. This stoichiometry was confirmed by the tracer experiments of Mehler and Brown (9).

If catalase and ethanol were omitted and the chloroplasts illuminated without addenda, no net oxygen consumption was observed. However, using labeled oxygen in this system Brown and Good (1) showed that the chloroplasts consumed oxygen from the milieu while at the same time they produced an equivalent amount of molecular oxygen. Both processes were shown to be light dependent. In effect, chloroplasts are able to promote an "oxygen exchange" between the oxygen dissolved in the suspending medium and the oxygen combined as water. The one for one stoichiometry of this exchange reaction could be explained most simply by assuming that the endogenous catalase present in the chloroplasts is sufficient to decompose the peroxide (formed as a product of oxygen reduction) to water and 0.5 molecule of oxygen. This explanation may or may not be correct because reduction of oxygen to water rather than to H_2O_2 has not been ruled out. According to the former scheme, which will be assumed to be correct, in the exchange reaction each mole of oxygen reduced by chloroplast preparations in the light is exactly balanced by the production of 0.5 mole from the (OH)-product of photolysis and 0.5 mole from the decomposition of peroxide.

Mehler (8) found that in the presence of catalase and ethanol the rates of net oxygen uptake by chloroplasts in the light were considerably enhanced when these preparations had previously reduced quinone (Hill reaction). These enhanced rates of net oxygen uptake could be increased still further upon addition of ascorbic acid to the reaction system. These observations were confirmed by Good and Hill (3) and also by Habermann and Brown (4).

In the present paper it will be shown that following quinone reduction by chloroplast preparations the rate of the exchange reaction is enhanced in a manner identical in nature to the stimulated Mehler reaction. The enhanced rate is the result of an overall stimulation with no change in reaction stoichiometry. The isotopic tracer method using oxygen labeled with its mass 18 isotope and the mass spectrometer were used to determine the nature of the stimulatory effects of quinone on these reactions.

MATERIALS AND METHODS

Chloroplast preparations were made from the mature leaves of either greenhouse- or field-grown *Phytolacca americana* L. (pokeweed). Leaves were homogenized for three minutes in 0.5 M sucrose in a Waring blendor. The homogenate was strained through several thicknesses of cheesecloth and centrifuged at $1000 \times G$ for 10 minutes in a Servall refrigerated centrifuge. The supernatant was then centrifuged for 20 minutes at $20{,}000 \times G$. Temperature of the rotor during both centrifugations was 0° C. The centrifugate containing intact and fragmented chloroplasts was resuspended in a medium made up of M/15 phosphate buffer (pH 6.8) 0.5 M in sucrose and 0.05 M in KCl. As far as possible all operations were carried out in the dark at temperatures close to 0° C. The prepared chloroplasts were kept frozen in a deep freeze at −33° C until needed. For use, the suspensions were diluted with the above medium so that they contained the desired concentration of chlorophyll.

Conventional rectangular Warburg vessels were used for both manometric and mass spectrometric experiments. In the former the temperature was 18.8° C, in the latter 23° C. Adaptation of the mass spectrometer (Consolidated Engineering Company Model 21-201) for measurement of metabolic gas exchange has been described by Brown, Nier and Van Norman (2) and further modifications of the apparatus have been described by Johnston and Brown (5). Reaction vessels were illuminated either by a bank of photoflood bulbs placed below or by white fluorescent tubes mounted behind the bath. In the latter case an inclined mirror was so placed that light was reflected onto the bottoms of the vessels. Incident light intensities up to approximately 500 ft-c could be obtained from either source.

The gas phase in all manometric experiments was air. No absorbant for CO_2 was used. CO_2 tension

[1] Received January 8, 1958.

[2] The contents of this paper are a part of a thesis submitted to the graduate faculty of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree.

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was measured during many comparable experiments using the mass spectrometer and no evidence was found for pressure changes caused by CO_2 in any of the chloroplast reactions studied. In experiments with the mass spectrometer the gas phase was a mixture of oxygen of mass 32 and mass 34 in helium. The vessel containing 3 cc of buffered chloroplast suspension plus addenda was first flushed with helium, then oxygen enriched with mass 34 ($O^{16}O^{18}$) was added to the desired concentration of total oxygen (usually 1.5 to 3 %).

Quinone was purified by sublimation of the commercially available chemical. Crystalline catalase (General Biochemicals, Inc.) was used without further purification.

Results

Influence of Pretreatment with Quinone: The rate of oxygen uptake following the reduction of a given amount of quinone was found to depend on whether quinone was added to the reaction mixture before or after the beginning of illumination. In view of this dependence of the extent of stimulation on procedure, standard protocol had to be followed. The reaction mixtures containing quinone were equilibrated in the dark for 10 to 20 minutes before the beginning of illumination to insure maximum stimulation. A standardized procedure did not eliminate variations due to the nature of the different chloroplast preparations. For a given batch of chloroplasts, however, rates were consistent and reproducible.

Dependence of Rates on Initial Quinone Concentration: Figure 1 shows the net oxygen time courses in vessels with varied amounts of quinone added to mixtures of chloroplasts, catalase and ethanol which were otherwise identical in composition. In figure 2 the net rates of oxygen uptake in the Mehler reaction following quinone reduction are plotted against initial quinone concentration. In general, maximally stimulated rates were obtained when the initial quinone concentrations in the reaction mixtures were at least 10^{-3} molar. These observations confirmed with chloroplast preparations from a different source the original observations of Mehler (8).

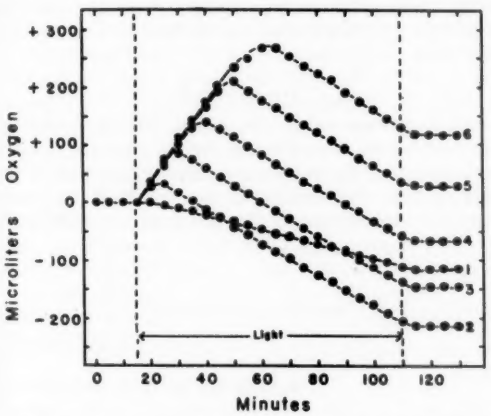

Fig. 1. Time courses of net oxygen production and consumption in vessels containing varied amounts of quinone added to mixtures of chloroplasts, catalase and ethanol of otherwise identical composition. Vessel contents: 3 cc chloroplast suspension (0.4 mg chlorophyll), 4 mg catalase, 0.1 cc 50 % ethanol, quinone as follows: 1. No quinone, 2. 5.5 micromoles, 3. 11.1 micromoles, 4. 16.7 micromoles, 5. 22.2 micromoles, 6. 27.8 micromoles.

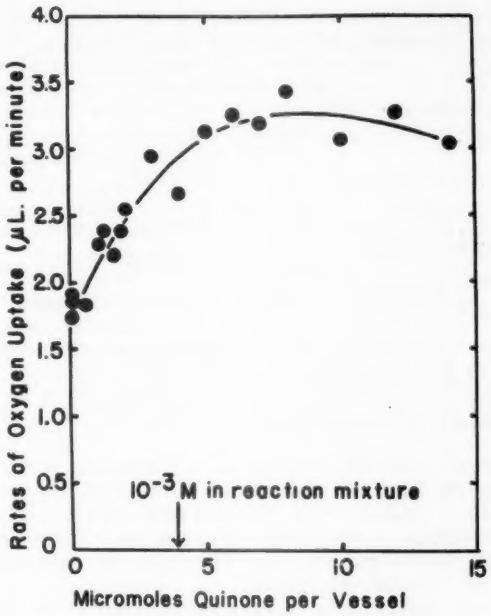

Fig. 2. Effect of initial quinone concentration on rates of net oxygen uptake after completion of quinone reduction. Vessel contents: 3 cc chloroplast suspension (0.4 mg chlorophyll), 4 mg catalase, 0.1 cc 50 % ethanol, 0 to 14 micromoles quinone.

From the known Mehler reaction stoichiometry one might expect that the rates of net oxygen uptake in this reaction would equal the rates of oxygen production with other Hill reagents such as quinone. When maximally stimulated by quinone, rates were usually 2 to 3 times those of the unstimulated Mehler reaction. Even when maximally stimulated, however, observed rates were rarely more than 50 % of those expected from measured Hill rates with identical chloroplast preparations.

Experiments with the Mass Spectrometer: Manometric measurements tell us nothing about the nature of the quinone stimulation illustrated in figure 1, i.e., whether increased net rates are caused by an acceleration of oxygen uptake, an inhibition of production, or changes in the rates of both. The nature of the enhanced rates of net oxygen uptake observed manometrically had not previously been investigated. The effect of quinone reduction on the exchange re-

TABLE I

EFFECTS OF QUINONE REDUCTION ON RATES OF OXYGEN UPTAKE AND PRODUCTION IN THE MEHLER SYSTEM

	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	NET RATE (μL/MIN)
Unstimulated	1.33 ± 0.33	0.64 ± 0.14	−0.75 ± 0.24
Quinone reaction	0.12 ± 0.18	2.97 ± 0.27	+2.86 ± 0.33
Quinone stimulated	3.19 ± 0.19	1.42 ± 0.21	−1.79 ± 0.08
Percentage of unstimulated rate	239 %	222 %	238 %

Vessel contents: 3 cc chloroplast suspension (containing 0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 micromoles quinone (in those vessels containing quinone). Means and standard deviations of the means represent 6 determinations.

action could not be examined manometrically because in this reaction no net pressure changes are observed. Data obtained with tracer oxygen and the mass spectrometer showed that the exchange reaction and the Mehler reaction are accelerated in the same way following quinone reduction.

a. Effects of previous quinone reduction on the Mehler reaction: Table I summarizes the results of 12 experiments. In each experiment the vessel contained 3 cc chloroplast suspension (0.21 mg chlorophyll), 0.1 cc 50 % ethanol and 6 mg catalase. In half the experiments 6 micromoles quinone were present in the reaction mixture (equilibrated in the dark for 20 minutes before the beginning of illumination); in the other half, no quinone was present in the vessels.

The average rate of oxygen uptake after quinone reduction was 239 % of the unstimulated rate, while the rate of oxygen production was 222 % of the unstimulated rate. Net quinone-stimulated rates were 238 % of the unstimulated rates of net oxygen uptake. Thus, enhanced rates of net oxygen uptake following quinone reduction appeared to result from an overall stimulation, i.e., an enhancement of rates of both uptake and production with no change in the stoichiometry of the Mehler reaction.

b. Effects of previous quinone reduction on the exchange reaction: The exchange reaction was accelerated by previous reduction of quinone by illuminated chloroplasts and, as with the Mehler reaction, the extent of the stimulation was enhanced by a period of dark equilibration of the quinone with the chloroplast preparation (see table II). In experiment 1 quinone was added to the chloroplast preparation after a period of exchange in the light. Chloroplasts and quinone were left in the dark for 10 minutes after the addition prior to the next period of illumination. The rates of oxygen uptake and production (after the quinone had been reduced in the light) were about twice those observed in the light period preceding the addition of quinone. A period of illumination following another dark period showed that the rates did not revert to unstimulated values. In experiment 2 quinone was tipped from the sidearm while the unstimulated exchange reaction was proceeding normally in the light. Quinone reduction, of course, occurred immediately, followed by the stimulated oxygen exchange.

The data in table II are typical of the many experiments which were run. The chloroplast preparations used in these two experiments were not identical and because of the difference in activity of the two preparations comparison of rates is uncertain. Increases in the rates of both uptake and production of oxygen were observed in experiment 2 (112 % and 120 % of unstimulated values). However, the extent of stimulation was only about 0.1 of that observed in experiment 1 where 192 % and 201 % of the unstimulated values were observed following quinone reduction by chloroplasts equilibrated with quinone in the dark.

DISCUSSION

Experiments using the tracer oxygen technique have indicated that the stimulatory effect of quinone reduction on the subsequent net oxygen uptake by illuminated chloroplasts in the presence of excess catalase and ethanol is the result of a simultaneous

TABLE II

EFFECTS OF QUINONE REDUCTION ON RATES OF OXYGEN UPTAKE AND PRODUCTION IN THE EXCHANGE REACTION

	1. QUINONE TIP FOLLOWED BY 10 MINUTES DARK		2. QUINONE TIPPED IN THE LIGHT	
	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)
Unstimulated exchange	2.03	1.85	3.26	2.92
Quinone reaction	0.00	4.15	0.00	5.64
Quinone stimulated exchange	3.90 *	3.72 *	3.65	3.52
Percent of unstimulated rates	192 %	201 %	112 %	120 %

* Average for two light periods.

Vessel contents: 1. 3 cc chloroplast suspension (0.94 mg chlorophyll), 6 micromoles quinone in sidearm. 2.3 cc chloroplast suspension (0.70 mg chlorophyll), 6 micromoles quinone in sidearm.

increase in the rates of both uptake and production of oxygen. An effect, identical in nature, on the related exchange reaction was observed. In both cases quinone reduction which so greatly enhanced reaction rates produced no change in the stoichiometry of the overall reaction.

Although these experiments have further clarified the nature of the quinone stimulation, the mechanism by which this substance acts is still a matter of conjecture. The basis of the net effect observed in the Mehler system is of course the exchange reaction. Thus the quinone effect has to be explained in a way valid for both the exchange system (incorporating the catalatic decomposition of H_2O_2) and the Mehler, or catalase-ethanol-containing system (in which H_2O_2 is removed by the coupled oxidation of ethanol to acetaldehyde). Any explanation also has to take into account the fact that the stoichiometry, i.e., the ratio of oxygen taken up to oxygen produced, does not change.

Since it is a common experience with the photochemical system in plants that whatever happens to the reducing side is reflected in a corresponding change on the oxygen side, we have the following choices of mechanism: (a) The product of quinone reduction may inhibit the back reaction between photochemically produced (H) and (OH) and thus favor the reduction of free oxygen; (b) it may stimulate the evolution of oxygen and thus automatically release more (H) for reduction; and (c) it may catalyze the formation of peroxide from (H) and free oxygen which would as in (b) lead to a proportionately greater rate of oxygen production.

Mehler (8) showed that no stimulation of oxygen uptake resulted when hydroquinone was used in place of quinone. Therefore a simple catalytic reaction in solution based on a shuttle between hydroquinone and quinone on either side of the exchange system is not a sufficient explanation for the observed stimulation.

Quinone must be reduced in the presence of chloroplasts. Because of the enhancement of the quinone effect by equilibration with chloroplasts in the dark prior to the beginning of illumination (and quinone reduction), it seems appropriate to assume that there may be a binding of the quinone at a reactive site on the chlorophyll complex. This still does not allow us to decide, however, whether its effect consists of an inhibition of back reactions or the catalysis of hydrogen transfer.

Summary

Illuminated chloroplasts form hydrogen peroxide in the presence of oxygen by a photoreduction analogous to that of iron salts, quinone and other Hill reagents. Such a reduction of oxygen is accompanied by the photochemical evolution of oxygen from water. Labeling of the molecular oxygen with O^{18} has been the means for distinguishing between uptake and release of this gas and this method has been employed again in the present study.

Previous observations that the reduction of small amounts of quinone by illuminated chloroplasts accelerated net oxygen uptake in the Mehler system have been confirmed. It has been shown that stimulation by quinone does not change the ratio of uptake to production of oxygen. This holds for the Mehler system and for the simpler oxygen exchange system as well.

Possible mechanisms for the quinone effect have been discussed.

The author wishes to thank Prof. Allan H. Brown for his help and encouragement during the course of experimentation. These studies were aided through funds supplied by the Graduate School of the University of Minnesota and also by a contract between the Office of Naval Research, Department of the Navy and the University of Minnesota (NR 104 030). Tracer oxygen was obtained from Prof. A. O. C. Nier and was prepared under a grant from the American Cancer Society, through the Committee on Growth of the National Research Council. During the summer of 1955 this work was encouraged by the financial assistance of the Lawrence S. Moyer fellowship. It is with deep gratitude that this support is acknowledged.

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QUANTITATIVE ASSAY OF CHLOROGENIC ACID AND ITS PATTERN OF DISTRIBUTION WITHIN TOBACCO LEAVES[1]

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In order to relate the composition of the green tobacco leaf to the type of reactions which occur when leaves are harvested and cured, it was desirable to measure the concentration of the phenol, chlorogenic acid, in disks cut from fresh leaves. Studies on wheat (7, 10) have shown that the concentration of substances synthesized in situ such as proteins and chlorophyll is not uniform throughout the leaf but is higher at the tip than at the leaf base. A similar tip to base gradient has been found in the concentration of total alkaloids of the tobacco leaf (2), most of which enter the leaf by translocation. Ascorbic acid but not carotene concentration follows this gradient pattern in turnip leaves (5). On the other hand, no apparent gradient of ascorbic acid was found in the apple leaf (3).

Hence, analysis of the distribution of chlorogenic acid in mature tobacco leaves was undertaken so that a representative sample could be obtained without destroying the whole leaf. A quantitative colorimetric assay based on Hoepfner's original method (8) was devised for this purpose. To determine whether the definite pattern of chlorogenic acid distribution observed was a result of the pattern of translocation or represented actual physiological differences between various areas of the leaf, studies were made of the net synthesis of chlorogenic acid by various parts of the tobacco plant.

MATERIALS AND METHODS

The pattern of chlorogenic acid distribution was determined in fully expanded leaves of *Nicotiana tabacum*, var. Conn. 49, shade tobacco grown outdoors under a cheesecloth shade tent according to commercial practices (1). Leaves from the 12th and 18th nodes of three plants were harvested, washed and allowed to stand for an hour with their bases in water. Five disks, 27 mm in diameter, were punched from the lamina on each side of the midrib of the turgid leaves and weighed. The disks were taken from the tip, middle and base of each leaf at a position either near the midrib or the margin. The distance, along the midrib, of each disk from the tip of the leaf was measured on paper tracings of the sampled leaves.

CHLOROGENIC ACID EXTRACTION AND ASSAY: Each weighed disk was placed in 5 to 10 ml of boiling distilled water and held at 100° C in a water bath for 5 minutes. The boiled disk and its extracting solution then was either frozen for convenience or was transferred with water washes, after cooling, to a 15-ml glass homogenizer and ground mechanically. The suspension was boiled again for 5 minutes to insure complete extraction, cooled and diluted to 25 ml with washes from the homogenizer. This diluted sample was thoroughly mixed and filtered, and the filtrate was stored frozen until assayed.

Chlorogenic acid added to leaf tissue was recovered quantitatively when subjected to this procedure.

The Hoepfner reaction (8) is based on the fact that chlorogenic acid reacts with nitrite and acetic acid to produce a yellow color. Addition of alkali then converts the yellow complex to a bright red pigment. This reaction has commonly been employed only as a spray reagent for chlorogenic acid chromatograms (11). Our attempts to use Hoepfner's assay or various modifications of it gave erratic results initially. It was found that if chlorogenic acid was first absorbed on alumina, the assay became much more stable. Consequently the use of small columns of alumina was adopted.

Five to 10 ml of an extract prepared as described above and containing 0.1 to 1 micromole of chlorogenic acid was added to a column of chromatographic alumina previously washed with water. The chlorogenic acid, absorbed at the top as a yellow band, was washed with 5 ml of water, and a freshly mixed solution containing 2 ml of 0.5 % sodium nitrite and 2 ml of 5 % acetic acid was added. The yellow band became orange or tan as the nitrous acid mixture drained through. After washing with water again, 5 ml of 5 *N* sodium or potassium hydroxide was added to the column and the orange pigment at the top changed to bright red and moved down the column as a somewhat diffuse band. The alkaline eluate was collected and enough water was added to the column to bring the total volume of the eluate to 10 ml. After mixing, the absorbance of the red solution was measured at 525 mμ against a water blank with a Coleman model 8 colorimeter and followed the Beer-Lambert laws over the range mentioned above. The actual absorption peak of the red color occurred at 520 mμ. The column could be used again after washing it with 50 ml of water.

This reaction is given by a number of substances other than chlorogenic acid. Caffeic acid, for instance, gives one fifth the absorbance on a molar basis under the above conditions. However, as Hoepfner showed (8), caffeic acid turns red with nitrous acid alone and can be detected even in the presence of much greater quantities of chlorogenic acid. Rutin, another tobacco phenol, does not interfere with the assay.

Other cinnamic acid derivatives and some dihydroxy phenols, not usually found in tobacco, also react in the Hoepfner test (4). Consequently, application of this assay to plant extracts should be supplemented by chromatographic study.

[1] Received January 20, 1958.

Dawson and Wada (6) found two substances in addition to chlorogenic acid that reacted with the Hoepfner spray reagents on paper chromatograms of Conn. 49 tobacco. They showed that these substances must be very closely related structurally to chlorogenic acid, however. Chromatography of the extracts from plants used here indicated that chlorogenic acid accounted for 80 % of the reactive material eluted from the chromatograms. The aqueous phase of the *n*-butyl acetate, acetic acid, water mixture used by Dawson and Wada was employed as a solvent.

Except where noted, plants used for the study of chlorogenic acid synthesis were grown during the winter in the greenhouse. Net synthesis was measured in tissue taken from the plants, placed in Petri dishes on moist filter paper and maintained 24 hours under 400 to 500 ft-c of constant light at 23 to 25° C. Roots were washed thoroughly before being placed in culture. Dry weights were determined on aliquots of the boiled, diluted suspensions.

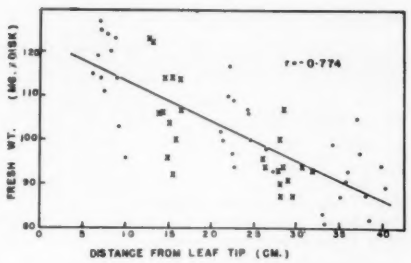
Results

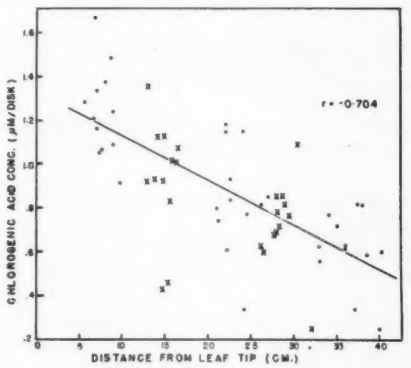
The fresh weights of leaf disks varied considerably with their positions in the leaf. Figure 1 shows that the weights of the disks decreased with increasing distance from the tip of the leaf. The slope of the regression line was – 0.905 mg/disk × cm and the correlation coefficient, r, was – 0.774. Thus, for leaves 40 cm long the fresh weight of disks from the tip averaged 35 mg heavier than disks from the base, a difference of 30 to 40 %.

The pattern of chlorogenic acid distribution in the leaves was similar to the fresh weight changes (fig 2). Again the highest concentrations per disk were from tip samples and the lowest from the base. The chlorogenic acid content of the tip samples was on the order of 1.2 micromoles per disk, about twice the content of basal disks. The regression of chlorogenic acid content on distance from the leaf tip yielded a line with a slope of – 0.00362 micromoles/disk × cm. The correlation coefficient was – 0.702.

The differences in chlorogenic acid content were not merely a reflection of differences in fresh weight for the ratio of chlorogenic acid to fresh weight also changed (fig 3). With increasing distance from the leaf tip the concentration of chlorogenic acid per unit fresh weight decreased significantly. The slope of the regression line was – 0.124 micromoles/g × cm and a correlation coefficient of – 0.55 was obtained. The mean chlorogenic acid concentration was 0.3 % of the fresh weight and 3 % on a dry weight basis.

Attempts were made to locate the sites of synthesis of chlorogenic acid in the plant by measuring the net change in chlorogenic acid concentration in disks of leaf lamina, 1-mm-thick stem sections, and in excised roots after 24 hours of culture. Table I shows the results of a number of such experiments. Laminar tissue from both field and greenhouse grown plants synthesized chlorogenic acid. Under the conditions of the experiments, the net amounts formed were similar even though the field grown leaves contained two to three times as much chlorogenic acid initially. Culture of disks in the dark rather than in the light did not lead to a net synthesis indicating that light is an important factor in the process. Disks maintained for more than 24 hours usually did not show any further increase in chlorogenic acid. Substitution of whole, excised leaves for leaf disks in





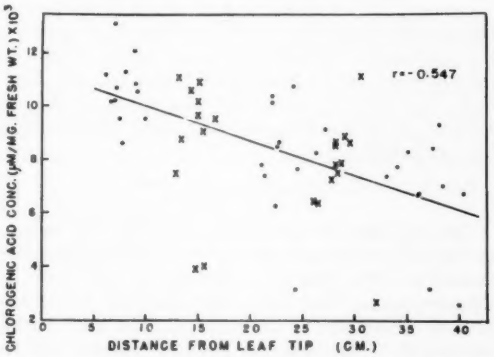


Fig. 1 (*top*). The fresh weight of 5.73 cm² disks punched from various positions in the leaf: closed circle, disks punched closer to the midrib than the margin; X, disks punched closer to the margin than the midrib of the leaf.

Fig. 2 (*middle*). The chlorogenic acid content of 5.73 cm² tobacco leaf disks. Positions of disks same as in figure 1.

Fig. 3 (*bottom*). The concentration of chlorogenic acid per mg fresh weight of leaf disks. Positions same as in figure 1.

TABLE I

NET SYNTHESIS OF CHLOROGENIC ACID IN TOBACCO TISSUE

MATERIAL	MICROMOLES OF CHLOROGENIC ACID *		MICROMOLES SYNTHESIZED
	INITIAL	24 HOURS	
		per g original fresh weight	
Leaf disks from field grown plants	12.80	13.80	1.00
Leaf disks from greenhouse plants	5.30	6.44	1.14
Leaf disks from greenhouse plants (cultured in the dark)	5.30	5.45	0.15
		per g original dry weight	
Leaf disks from greenhouse plants	52.7 (7.42)	61.8 (8.70)	9.1 (1.28)
Stem sections from greenhouse plants	12.5	21.5	9.0
Excised roots from greenhouse plants	15.6	12.9	-2.7

Figures in parentheses express the chlorogenic acid concentration on a fresh weight basis.
* Values represent data obtained from at least two plant samples.

the above experiments yielded approximately the same results.

Data in table I also demonstrate that stem tissue as well as the leaf lamina could synthesize chlorogenic acid. Roots, on the other hand, showed no net increase at all. On a dry weight basis, both stem and root tissue contained only one fourth the concentration of chlorogenic acid found in leaves.

Attempts were made to demonstrate direct physiological differences within the leaf itself by studying the synthesis of chlorogenic acid in disks from both the tip and base of greenhouse grown leaves. However, no consistent difference was found in the net amount of chlorogenic acid synthesized by disks from the two areas.

DISCUSSION

The gradient of chlorogenic acid from tip to base of the leaf correlates well with the visual gradients of change in color that occur during the air curing of tobacco leaves. The tip is the first part of the leaf to turn brown and is often the darkest after curing has ceased. These observations may be a direct result of the chlorogenic acid gradient, for this phenol appears to be involved in browning (9, 12).

Since there could be no translocation from other parts of the plant, the net increase in chlorogenic acid observed in isolated leaf disks clearly indicates that this compound can be synthesized in leaf tissue. Such findings suggest that the tip to base gradient may result from actual physiological differences between various areas of the leaf. However, since the data also show that chlorogenic acid is found in the stem and roots and can be synthesized by stem tissue as well, the possibility exists that chlorogenic acid is translocated in and out of the leaf.

Dawson and Wada (6) have reported considerably lower values for chlorogenic acid concentration in the variety of tobacco analyzed here. The differences in their values and those reported above could, in part, result from the fact that the field tobacco used in this study was grown during an exceptionally dry summer.

SUMMARY

A rapid, quantitative, colorimetric method of chlorogenic acid estimation involving the use of the Hoepfner reagents has been described. The method was used to assay chlorogenic acid in small disks of green tobacco leaves and is selective but not specific.

A distinct gradient of chlorogenic acid was found from tip to base of the tobacco leaf. The amount of chlorogenic acid in leaf disks was greatest at the leaf tip and decreased with distance toward the base.

Chlorogenic acid was found in stem and root tissue also although the concentration was one fourth that of leaf tissue. A net synthesis of chlorogenic acid occurred in leaf disks cultured in the light but not in the dark. Stem sections but not excised roots also showed net synthesis of chlorogenic acid.

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ALGAL GROWTH IN CROSSED GRADIENTS OF LIGHT INTENSITY AND TEMPERATURE[1,2]

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Two of the major factors that determine the growth responses of plants are the light intensity and the temperature. The effect of either of these variables on the growth rate is strongly influenced by the level of the other factor. A complete study of just these two variables, even for a single species, is a large-scale job.

The apparatus described here provides a visual picture of algal growth as a function of two factors varying along rectangular coordinates.

The principle is to use a thin layer of agar with algae growing on its surface. The agar layer is provided with a temperature gradient from left to right and a light intensity gradient from front to back. There is, at any given intensity, a range of temperature, and at any given temperature a range of intensities. Growth response is observed visually or photographed at intervals, thus adding the dimension of time.

Visual interpretation of algal growth response to the crossed gradients of light intensity and temperature are subjective and only roughly quantitative. Precision of the response estimate is improved if response is measured, for example, in terms of pigment formation (1, 2).

This paper gives details of construction and operation of the device which has been used to determine growth patterns of several unicellular algae (3, 4).

APPARATUS

The base of the crossed gradient culture chamber is a 12×16 inch aluminum plate $\frac{3}{4}$ inches thick. The $11\frac{3}{8} \times 12$ inch center part of the plate, the actual growth chamber, is bounded by metal edges $\frac{1}{8}$ inch high and is painted with chemically inert white Tygon. A Lucite spacer resting on the ridge surrounding the growth chamber supports the cover, a shallow Lucite box, through which warm water is circulated to prevent condensation of water. Figure 1 shows these parts in cross section.

The aluminum base plate extends 2 inches on either side of the growth chamber. On each side transverse borings carry water from the back of the plate to the front and again through the plate to a rear exit port. The left edge of the plate is kept uniformly cold and the right edge at a high temperature by continuous circulation of water from two constant temperature baths. The flow of heat through the aluminum produces a temperature gradient across the plate, which is mounted on cork for thermal insulation. The cork lined frame is supported on leveling screws.

The light comes from three 300-watt projector spot lamps run from a voltage regulator. Just below the lamps is a Lucite tank containing a 4.5-inch-deep heat filter of distilled water supplied from a reservoir with an automatic level device.

The intensity gradient on the culture surface is provided by directing the projector spot lamps toward the rear of a ground-glass plate, 4 inches above the agar surface. There is also a comb-shaped piece of translucent paper cut to appropriate shape by successive trials. The comb is on top of the ground-glass and is protected by a clear-glass plate. In addition to these arrangements for producing the intensity gradient, there is an opaque shade spaced between the ground-glass diffusing surface and the growth chamber. The arrangement of these parts is shown in figures 1 and 2.

Five percent CO_2 in air enters the growth chamber through 6 ports spaced along its front edge. Before entering these ports the gas stream, regulated by separate needle valves, bubbles through water in individual test tubes. To prevent the agar from drying out, the water is electrically heated to 80° and 90° C respectively in the tubes which saturate the gas entering the two high temperature ports.

[1] Received February 3, 1958.

[2] Dedicated to Professor Richard Harder, University of Göttingen, on the occasion of his 70th birthday, March 21, 1958.

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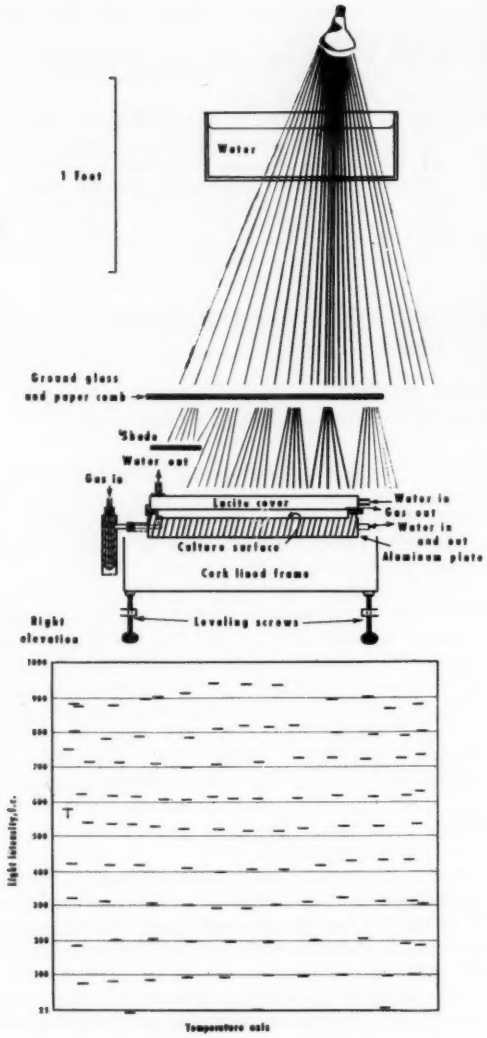

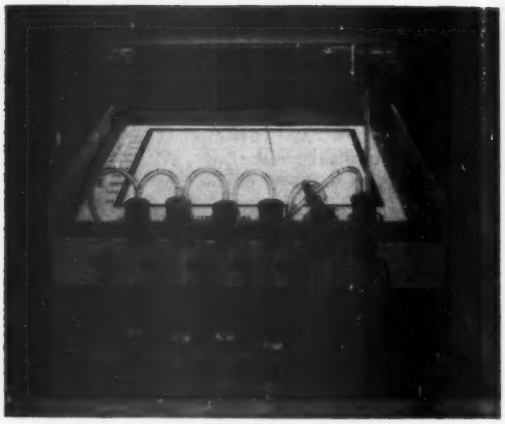

FIG. 1 (*top left*). The crossed gradient culture apparatus. (Cross section from right-hand side.)

FIG. 2 (*top right*). Front view of crossed gradient culture apparatus as arranged for photographing the culture. The ground glass screen with its paper comb, the shade below it, and the Lucite box cover have been pushed back on racks and the printed cardboard scale put in place. The culture surface is within the black lines.

FIG. 3 (*bottom left*). Actual intensity contours as measured over the surface of the culture plate (dashes), compared with the nominal intensity (full lines).

OPERATING PROCEDURE

The temperature is checked by a thermocouple. The measuring junction may be taped to the plate, or inserted in the agar layer.

To check the light intensity gradient the aluminum plate is removed and the Lucite cover is held in its normal place with appropriate supports. A photographic exposure meter is mounted on a frame which holds its sensitive surface in the plane normally occupied by the agar surface. This frame rests on the base of the apparatus over a sheet of paper and provision is made in the frame for marking the paper directly under the center of the photosensitive surface. To adjust the intensity gradient 10 lines are ruled across the paper representing even intervals from the front to the back of the culture surface. These lines are labeled from 0 to 1000 ft-c. Trial adjustments of the lamp position and of the shape of the paper comb are made and the desired intensity at various places is compared with the actual reading of the exposure meter. Separate variac controls of the individual lamp intensities are made and the lamp positions adjusted. After a reasonable match of the desired and the actual intensities is achieved the final intensity contours are plotted by moving the photocell to those positions which give exact readings of each 100 ft-c on the scale and marking the paper accordingly. These marks at 100 ft-c intervals give a contour plot of the actual intensity. An illustration of such a plot is given in figure 3.

Before pouring the agar, the plate is leveled and then heated electrically to above the solidifying temperature of agar. The agar (200 ml), containing appropriate nutrient salts, is poured on to give a layer about 2 mm deep. After the agar has cooled the plate is inoculated.

Spraying and painting proved to be impractical inoculation procedures. The most successful way is to pour on a layer of liquid culture medium containing the suspended algae and then allow the plate to dry overnight with the cover off. This gives a uniform inoculum and the excess water either evaporates or is taken up in the agar.

In some cases it is better to illuminate the plate at room temperature with weak uniform light to start growth. After the inoculum has started grow-

ing uniformly on the plate, the temperature and intensity gradients are set up.

Photographs are taken under identical conditions once or twice a day. The camera is permanently mounted and four photoflood lamps are fixed to the side walls of the surrounding chamber to give reproducible and uniform illumination. These lamps are used only for photography. To take pictures it is necessary to slide the water tank, the Lucite water box cover, and the ground-glass plate out of the way on tracks with rollers. A white cardboard frame printed with the experimental data is put around the plate before taking pictures.

To make quantitative measurements of the pigment formation from some of the algae, small pieces of the agar were removed and placed directly in the spectrophotometer with opal glass plates. The results of this work with blue-green algae are described elsewhere (1, 2).

The most serious difficulties in operation were drying out of the agar and achieving a uniform inoculation. Contamination of the algal cultures caused very little trouble because of the largely inorganic media, the abundant inoculum, and the short duration of the runs.

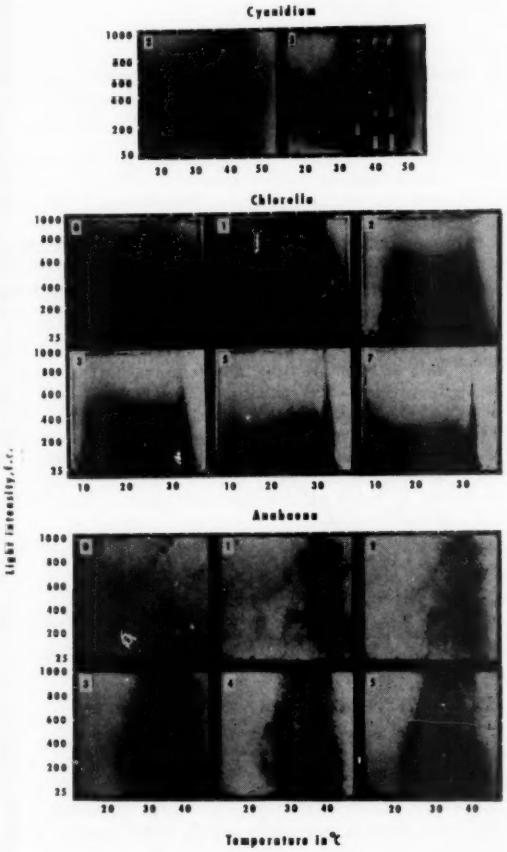

FIG. 4. The growth of algae on the crossed gradient plate. The days elapsed since inoculation are given on each photograph.

TABLE I

LIGHT INTENSITIES AND TEMPERATURES FOR BEST GROWTH OF ALGAE AS OBSERVED ON THE CROSSED GRADIENT PLATE

SPECIES	AGE HRS	APPROX LOWER LIMIT	OPTIMUM	APPROX UPPER LIMIT
Chlorella pyrenoidosa	24	22 50	*29* *250*	33° C 400 ft-c
Anabaena sp.	48	28 150	*36* *400*	43° C 1000 ft-c
Anacystis nidulans	12	36 Equal growth at all intensities from 25 to 1000 ft-c	*40*	43° C
Cyanidium caldarium	48	38 100	*43* *350*	47° C 700 ft-c
Haematococcus lacustris		12 50	Growth over entire range of temperature and intensity	33° C 1000 ft-c

The following media solidified with 1.5 % agar were used: (A) Chlorella and Haematococcus: KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4 \cdot 7\,H_2O$, 1.0 g/L with 1 ml Hutner's trace element mixture. The inoculum of Haematococcus was grown at 5° C. (B) Anabaena and Anacystis: Myers and Kratz 1955 D medium. (C) Cyanidium: NH_4Cl, 0.535; $MgSO_4 \cdot 7\,H_2O$; 0.246; $CaCl_2$, 0.055; KH_2PO_4, 0.272 g/L; H_2SO_4 0.01 M with a trace of Fe and 1 ml Hutner's trace element mixture.

RESULTS

The pattern of algal growth on the gradient plate changes greatly in the course of a few days. The later patterns are much sharper and more characteristic than those seen at first. These striking final patterns have been described for most of the species so far cultivated. Only recently, however, has it become clear that the patterns of the older cultures are distorted by secondary effects and hence should not be taken to indicate the optimum growth conditions for the algae.

The complication of the secondary effects are best shown by the experiment with *Cyanidium caldarium* (fig 4). After two days this alga had produced a green zone within the range shown in table I. After five days most of this region was no longer green, but yellow, while the adjacent region, where the growth was poor at first, had become deep green. It appears that the early luxuriant growth had exhausted the nutrient supply in the optimum region so further growth there was impossible. The pattern produced later was therefore artificially distorted by

the nutrient deficiency. The apparent narrow temperature range of maximum growth found in the old cultures of Chlorella and Anacystis also seem to be caused by such secondary effects.

We have therefore prepared table I from the earliest clear pattern that developed. It should represent the optimum conditions for cultivating these algae.

In figure 4 the development of a *Chlorella pyrenoidosa* and of an Anabaena culture are also shown. Some difficulties in preparing a uniform inoculum of the Anabaena are evident in the figure but do not distort the conclusions appreciably.

With Haematococcus some growth was evident over the temperature range of 12 to 33° C and light intensities from 50 to 1000 ft-c with greater growth toward the high temperature side. At the higher intensities the color was red and shaded to brownish green at intensities below about 200 ft-c. The optima were broad and not clearly shown in this experiment.

Discussion

Crossed gradient growth studies should be useful in selecting appropriate algal strains for the temperature and intensity prevailing in large-scale algal farms. Studies of the behavior of mixed cultures on this crossed gradient plate would appear to be of value in showing how to adjust the conditions of a large-scale culture to give any desired strain a competitive advantage.

Another promising application of the crossed gradient plate would be as a means of isolating from nature algal strains having specified temperature and intensity tolerances.

The influence of light and temperature on growth of Lemna, a small higher plant, and on the germination of light-sensitive seeds, is being investigated with this device by Mrs. Brian J. Elliott. A number of other applications of the crossed gradient principle, using variables other than temperature and light, appear to be feasible. Several papers have appeared (5, 6) describing pH and chemical gradients in thin layers of agar. These gradients are established by pouring the plate slightly off level with half of the agar and then pouring an equal volume of a different composition with the plate tilted the opposite way. The plate here described would allow such a chemical gradient to be used on one axis with either temperature or light intensity on the other.

Summary

A device is described for growing algae on an agar surface having a temperature gradient from left to right crossed by a light intensity gradient from front to back. The optimum growth conditions for several algae have been determined with this apparatus.

The culture plate and Lucite parts were made by Mr. L. R. Kruger. The supporting frame, thermostats, and auxiliary components were made and kept in operating condition by Mr. R. W. Hart who also drew the illustrations. Several of the experimental runs were done by Miss Helen S. Huang. The algal cultures were kindly sent by Drs. Jack Myers, M. B. Allen and R. C. Starr.

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ABSENCE OF SEED DORMANCY IN A WHITE MUTANT STRAIN OF HELIANTHUS ANNUUS L.[1]

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The premature germination of seeds which normally require a period of dormancy has been reported in many plant species. As early as 1875 Ascherson (1) described apples which contained prematurely germinating seeds and in 1880 Treichel (13) reported observing germinating seeds within several kinds of fleshy fruits. About three decades ago several viviparous mutants of maize were described in papers by Mangelsdorf (7, 8, 9), Eyster (4, 5, 6) and

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Sprague (12). Mangelsdorf not only studied the genetic causes for such mutations but also commented on the apparent relationship between premature germination, endosperm color of the seed and the frequent lack of chlorophyll in the mutant seedlings. He suggested that the absence of chlorophyll in certain viviparous mutants might have resulted from the inhibition of its formation because of the premature germination of the embryos. Robertson (10), however, presented convincing evidence against this hypothesis based on studies of cultured excised corn embryos. In at least one class of viviparous mutants of maize there was an apparent lack of carotenoid formation in addition to abnormal chlorophyll development accompanying the expression of vivipary (10).

We have observed a similar lack of dormancy in sunflower seeds homozygous for the white albino mutation. This lack of dormancy seems to be correlated with the absence of any detectable amounts of the colored carotenoid pigments in the seedlings of this mutant. A yellow albino mutation which lacks carotene but contains xanthophyll has the normal requirement for a period of dormancy before the seeds will germinate.

These two albino forms were found in the progeny of ultrasonically treated normal seedlings of *Helianthus annuus* L., var. Russian Giant (14). Both mutations have bred as single recessive factors through many generations.

This phenomenon first came to our attention because of the high proportion of albino seedlings found in the plantings of freshly harvested seeds from self-pollinated heterozygous plants. Often 100 % of the resulting seedlings were albino and this was accompanied by a low percentage of seed germination. On storage the numbers of albino seedlings from batches of germinated seeds dropped to the expected 25 %, while the percent of green seedlings increased from 0 to 75 %. After storage for several months germination increased from low values to almost 100 %.

Table I summarizes the germination data of seeds from a series of heads of heterozygous green plants. Seeds from head number 1 were planted immediately after removal and without drying. The homozygous recessive (albino) seeds germinated uniformly and quickly. Ten days later the 41 ungerminated seeds were dug up. Ovary walls and seed coats were removed from 29 of these and the seeds were placed on moist filter paper in a Petri dish in weak light. One week later all had turned green and four had germinated slightly. The remaining 21 seeds were kept at 10° C for four months and then planted. All germinated to give vigorous green plants. Heads used in tests 2 through 6 were air dried at 20° C before seeds were removed from the head and planted. Heads used in test 2 were air dried for only one day and ray flowers were still in good condition at the time of shelling. All seeds in each head were planted. From the numbers germinated it is evident that in general the viability of these seeds was low.

While we have not observed germination of the white albino seeds in the head under field conditions, we have on several occasions removed heads while the ray flowers were still in good condition and embedded them in moist sand on the greenhouse bench. If kept moist for several days by covering with a bell jar or a piece of polyethylene embedded in sand at the edges, the white albinos germinated while the seeds remained in place in the head. The distribution of seeds homozygous for the white albino condition was random in the head. No green seeds germinated under these conditions.

It is not possible at this time to draw conclusions about the cause of this lack of dormancy in the white mutant seeds, although it is apparent that only seeds homozygous for this recessive mutation exhibit such premature germination. Two factors have been reported to be of importance in controlling the length of the period of dormancy of normal seeds of *Helianthus annuus* L.: (a) the presence of a chemical inhibitor of germination within the embryos of freshly harvested seeds (2, 3, 11); and (b) the specific temperature requirements of the embryo during the

TABLE I

GERMINATION DATA FROM SELFED HETEROZYGOUS GREEN HEADS AIR-DRIED FOR INCREASING PERIODS OF TIME AT 20° C

TEST NUMBER	HEAD NUMBER	NUMBER OF SEEDS PLANTED	SEEDLINGS				CONDITION OF SEEDS WHEN PLANTED
			GREEN	PERCENT	WHITE	PERCENT	
1	1	62	0	0	21	100	Directly from head, undried
2	2	260	3		39		
	3	160	0	3.9	20	96.1	Air-dried for 1 day at 20° C
	4	800	2		65		
3	5	260	12	20.7	44	79.3	Air-dried for 21 days at 20° C
4	6	520	91	42.7	122	57.3	Air-dried for 35 days at 20° C
5	7	320	130	76.5	40	23.5	Air-dried for 60 days at 20° C
6	Several heads used	...	7,450	75.2	2,459	24.8	Air-dried for 90 days at 20° C

TABLE II

EFFECT OF TEMPERATURE ON THE DORMANCY OF NORMAL GREEN SUNFLOWER SEEDS

TEST NUMBER	TEMPERATURE * (°C)	STORAGE TIME (WEEKS)	NUMBER OF SEEDS **	NUMBER OF SEEDLINGS	PERCENT GERMINATION
1	35	2	1600	140	15
		3	800	136	17
		5	725	136	19
		7	675	206	32
		10	625	538	86
2	24	2	1600	74	5
		3	800	222	28
		5	725	305	40
		7	675	531	73
		10	625	548	88
3	15	2	1600	50	3
		3	800	21	3
		5	725	36	5
		7	675	35	5
		10	625	544	87
4	0	2	1600	96	6
		3	800	258	32
		5	725	537	74
		7	675	492	73
		10	625	548	88

* Temperatures were controlled to within ± 1° C.
** All seeds used were selected to insure uniform viability. Maximum germination was about 90%. No further increase occurred after the 10th week.

"after-ripening" period (2). The length of dormancy in sunflower seeds (as in many other species) is dependent upon the temperature of storage after harvesting. The data in table II illustrate this effect of storage temperature on the length of dormancy of normal sunflower seeds.

By storing the seeds of selfed heterozygous green plants at a controlled temperature of 15° C it has been possible to prolong the dormancy of normal seeds so that negligible numbers of green seedlings germinated in plantings up to 7 weeks after harvesting. In this way it has been possible to obtain large numbers of white seedlings for experimental purposes without having to handle green ones.

The correlation of lack of dormancy with the complete absence of the colored carotenoid pigments in the resulting seedlings suggests the possibility that there is a close association of the genetic determiners of these factors on the chromosomes. Pigment analyses of normal and mutant seedlings have been run in several laboratories (Wallace and Schwarting (14); Habermann, University of Chicago, unpublished data; Gruen, Harvard University, private communication). No traces of any of the colored carotenoid pigments have been found in the white mutant seedlings. There is no correlation between this condition of premature germination and the ability to form chlorophyll. Both white and yellow mutants are able to form measurable quantities of chlorophyll during their seedling stages when they are grown under low intensity illumination (14).

SUMMARY

Seeds of *Helianthus annuus* L. homozygous for the white albino mutation exhibit a lack of dormancy similar to that reported in defective seeds of *Zea mays*. This premature germination seems to be associated with a carotenoid-free condition of the resulting seedlings. Seeds homozygous for the yellow albino mutation have the same dormancy requirements as heterozygous or homozygous green seeds. Storage of seeds harvested from selfed heterozygous green plants at controlled temperatures can prolong the dormancy of normal seeds so that large numbers of white seedlings can be grown for experimental purposes without the necessity for handling green ones.

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A COMPARISON OF CHLOROGENIC ACID AND CATECHOL AS SUBSTRATES FOR THE POLYPHENOL OXIDASE FROM TOBACCO AND MUSHROOM[1,2]

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It is reported that chlorogenic acid is widely distributed in plants (7) and is the principle polyphenol in tobacco leaves (8). When the tissues of the tobacco plant are injured, the polyphenols are oxidized and then polymerize, forming a brown pigment. This oxidation is reported to be catalyzed by polyphenol oxidase. Polyphenol oxidase has been identified in many species of plants and the enzyme from potato (3) and mushroom (2) has been investigated in some detail. In most cases the activity of the enzyme has been assayed by determination of the rate of oxidation of catechol using direct or indirect methods. Dawson et al (1) have presented curves indicating that rates of oxygen uptake in the presence of tobacco leaf extracts were greater when chlorogenic acid was used as a substrate than when either catechol or caffeic acid were utilized. These workers, however, did not indicate the concentrations of catechol or caffeic acid used. During the course of this investigation, it was found that the rate of oxidation of chlorogenic acid in the presence of a crude tobacco root extract was about 5-fold greater than the rate of oxidation of catechol. Conversely, the rate of oxidation of catechol catalyzed by a crude mushroom extract was about 6.5-fold greater than that with chlorogenic acid. The enzymes from both sources have been partially purified to determine whether or not more than one polyphenol oxidase was present in each of the extracts.

EXPERIMENTAL

ASSAY PROCEDURE: The catalysis of the oxidation of various polyphenolic substrates can be determined conveniently by the rate of disappearance of ascorbic acid in the presence of the enzyme and the substrate to be tested, provided that ascorbic acid oxidase is absent. In this procedure, the disappearance of ascorbic acid at 265 $m\mu$ is followed spectrophotometrically (6). The principle of the procedure is similar to that described by Miller et al (5). The essential difference is that the time for the oxidation of a measured quantity of ascorbic acid was recorded by Miller et al (5), whereas in the method to be described initial rates of ascorbic oxidation are measured spectrophotometrically.

The assay is carried out in a silica cuvette of 1.2 ml capacity. The reaction mixture in a final volume of 1 ml contains 0.1 M potassium phosphate buffer at pH 7.0, 5.7×10^{-5} M polyphenol (chlorogenic acid, catechol, dihydroxyphenylalanine and caffeic acid have been tested), 10^{-3} M EDTA (ethylenediaminetetraacetate) and 2.8×10^{-5} M ascorbic acid (obtained from a freshly prepared stock solution containing 0.1 M potassium phosphate buffer at pH 7.0, 10^{-3} M EDTA and 2.8×10^{-4} M ascorbic acid). Sufficient amount of extract is added to cause an OD decrease of 0.060 to 0.080 per minute. Enzyme activity is expressed as the OD decrease for the one-minute interval between 15 and 75 seconds after the reaction is initiated. Each extract is assayed for ascorbic acid oxidase activity by adding all reagents except the polyphenolic substrate. If any decrease in optical density occurs during a one-minute interval the polyphenol oxidase activity is corrected accordingly. The reaction rate is proportional to enzyme concentration over the range tested.

PURIFICATION OF EXTRACTS: The roots from tobacco plants (*Nicotiana Tabacum* L. var. Dixie Bright 101) grown in solution cultures were collected and placed in an Omni mixer (Ivan Sorvall Inc., Norwalk, Conn.) with a volume of cold 0.1 M potassium phosphate buffer at pH 7.0 equal in milliliters to the weight of the roots in grams. The entire purification operation was carried out at a temperature between 0 to 4° C unless otherwise indicated. The Omni mixer was operated at 50 % of the line voltage for a period of approximately 10 minutes. The major portion of the solid material was removed by straining the ground material through cheese cloth, and the liquid portion was centrifuged at $25,000 \times G$ for 20 minutes and the supernatant (referred to as fraction 1 in table I) was collected. Sufficient acetone at −15° C was added to the supernatant liquid until a concentration of 37 % by volume was obtained. The precipitate was collected by centrifugation and mixed with 15 ml of 0.1 M potassium phosphate buffer at pH 7.0. The denatured protein was removed by centrifugation at $12,500 \times G$ for 10 minutes and the supernatant (fraction 2 of table I) was collected. Sufficient solid $(NH_4)_2SO_4$ was added to fraction 2 to bring the concentration to 60 % saturation. After standing for 10 minutes the precipitated protein was collected by centrifugation at $12,500 \times G$ for 10 minutes and dissolved in 15 ml of 0.03 M potassium phosphate buffer at pH 7.0 (fraction 3 of table I). After dialyzing this fraction for four hours against two liters of 0.03 M potassium phosphate buffer at pH 7.0, the enzyme was adsorbed upon calcium phosphate gel. The adsorption was carried out stepwise by adding successively 2-ml portions of a 10 mg per ml gel suspension. After each gel addition, the suspension of gel and extract was mixed and then centrifuged and the supernatant tested for activity. This procedure was repeated until approximately 85 % of

[1] Received February 24, 1958.

[2] Contribution from the Faculty of Botany, North Carolina Agricultural Experiment Station and published with the approval of the Director as paper No. 891 of the Journal Series.

[3] E. G. Moss Predoctoral Fellow.

TABLE I

COMPARISON OF CHLOROGENIC ACID AND CATECHOL AS SUBSTRATES FOR TOBACCO ROOT POLYPHENOL OXIDASE AT VARIOUS DEGREES OF PURIFICATION

FRACTION	VOLUME	PROTEIN	SPECIFIC ACTIVITY *		S. A. WITH CHLOROGENIC ACID / S. A. WITH CATECHOL	RECOVERY
			CHLOROGENIC ACID	CATECHOL		
	ml	*mg/ml*	*units/mg protein*		*ratio*	*%*
1. Crude extract	170	2.9	8	1.7	4.7	...
2. 0 to 37 % acetone precipitate	15	6.0	53	8.8	6.0	92.0
3. 0 to 60 % $(NH_4)_2SO_4$ precipitate	15	2.0	70	17.0	4.1	18.0
Gel eluates						
4. Water	10	0.02	73	29.0	2.5	0.6
5. 1 % $(NH_4)_2SO_4$**	10	0.08	16	20.0	0.8	1.6
6. 3 % $(NH_4)_2SO_4$	10	0.03	156	60.0	2.7	1.9
7. 5 % $(NH_4)_2SO_4$	10	0.03	820	210.0	3.9	6.1
8. 10 % $(NH_4)_2SO_4$	10	0.01	1220	310.0	3.9	2.7

* The indirect method described in the text was used to assay the various fractions for activity. The concentration of either chlorogenic acid or catechol in the assay was 5.7×10^{-5} M. The protein content of extracts was determined by the method of Lowry et al (4).

** $(NH_4)_2SO_4$ solutions used for eluting the enzyme from the gel were adjusted to pH 8.0.

the enzyme was adsorbed as indicated by the assay method using chlorogenic acid as the substrate. The enzyme was eluted from the combined gel additions with water and with $(NH_4)_2SO_4$ solutions at pH 8 as indicated in table I. For the elution procedure, 10-ml portions of water or $(NH_4)_2SO_4$ solutions in the order indicated in table I, were thoroughly mixed with the gel and then the eluates collected by centrifugation.

The purification procedure for the polyphenol oxidase from mushroom (*Agaricus campestris* L.) sporophores was very similar to the procedure described for purifying the enzyme from tobacco roots. The essential difference in the two procedures was that two acetone precipitations were carried out with the crude extract from mushroom instead of one precipitation indicated for the purification of the tobacco root extract. The 37 to 55 % acetone precipitate of the mushroom extract (fraction 3 of table II) was dissolved in 0.03 M potassium phosphate buffer at pH 7.0, freed of denatured protein and fractioned with calcium phosphate gel by a procedure

TABLE II

COMPARISON OF CHLOROGENIC ACID AND CATECHOL AS SUBSTRATES FOR MUSHROOM POLYPHENOL OXIDASE AT VARIOUS DEGREES OF PURIFICATION

FRACTION	VOLUME	PROTEIN	SPECIFIC ACTIVITY *		S. A. WITH CHLOROGENIC ACID / S. A. WITH CATECHOL	RECOVERY
			CHLOROGENIC ACID	CATECHOL		
	ml	*mg/ml*	*units/mg protein*		*ratio*	*%*
1. Crude extract	220	6.0	4.7	31	0.15	...
2. 0 to 37 % acetone precipitate	15	4.5	22.0	90	0.25	15.0
3. 37 to 55 % acetone precipitate	15	10.6	25.0	150	0.17	60.0
4. 0 to 60 % $(NH_4)_2SO_4$ precipitate	15	3.5	56.0	595	0.09	40.0
Gel eluates						
5. Water	10	0.08	105.0	840	0.13	1.1
6. 1 % $(NH_4)_2SO_4$**	10	0.14	153.0	290	0.53	1.0
7. 3 % $(NH_4)_2SO_4$	10	0.14	69.0	580	0.12	2.0
8. 5 % $(NH_4)_2SO_4$	10	0.11	123.0	1360	0.09	3.5
9. 10 % $(NH_4)_2SO_4$	10	0.07	85.0	1200	0.07	2.0

* The indirect method described in the text was used to assay the various fractions for activity. The concentration of either chlorogenic acid or catechol in the assays was 5.7×10^{-5} M. The protein content of extracts was determined by the method of Lowry et al (4).

** $(NH_4)_2SO_4$ solutions used for eluting the enzyme from the gel were adjusted to pH 8.0.

identical with that described for fraction 2 of table I. The assay procedure with catechol as the substrate was used to determine the extent of adsorption of the enzyme on the gel.

SPECIFICITY FOR SUBSTRATES: The major purpose of carrying out the purification experiment reported in tables I and II was to determine the relative effectiveness of chlorogenic acid and of catechol as substrates for the various enzyme fractions.

It is apparent from the specific activities of the various tobacco root fractions (table I) that an overall purification of 153-fold was obtained in fraction 8 (10 % $(NH_4)_2SO_4$ eluate) when chlorogenic acid was used as a substrate and that a 182-fold purification was obtained for the same fraction when catechol was used as the substrate. As indicated by the ratios of the specific activities with these two substrates, the activities with chlorogenic acid generally ranged from 2.5- to 6-fold greater with chlorogenic acid than with catechol. Fraction 5, however, exhibited greater activity with catechol than with chlorogenic acid. From the variation in the ratios of activities with the two substrates it is apparent that tobacco roots contain more than one polyphenol oxidase.

From the data presented in table II, it is obvious that various fractions of mushroom extracts catalyze the oxidation of both chlorogenic acid and catechol. In contrast with the results obtained with the tobacco root fractions, the activity with catechol is considerably greater than that with chlorogenic acid. When chlorogenic acid was used as a substrate the greatest specific activity was obtained with fraction 6 which represents a 31-fold purification. When catechol was used as the substrate fractions 8 and 9 of the mushroom extracts exhibited the greatest specific activities and the purifications of these fractions were 44-fold and 39-fold respectively. It is apparent from the ratios of activities with the two substrates that the crude mushroom extract also contains more than one polyphenol oxidase.

In order to determine whether or not the difference in the rates of oxidation of chlorogenic acid and catechol was due to a difference in affinity for the enzyme, the K_M values were determined for the 5 % $(NH_4)_2SO_4$ gel eluates of tobacco (fraction 7, table I) and mushroom (fraction 8, table II). The values obtained for the tobacco extract were 6×10^{-4} M for catechol and 1.78×10^{-4} M for chlorogenic acid. It is apparent from these values that the affinity of the tobacco enzyme for chlorogenic acid is greater than that for catechol. When the mushroom extract was used as the enzyme a K_M value of 2.2×10^{-4} M was obtained with either catechol or chlorogenic acid as the substrate. By comparing this value with the value 6×10^{-4} M which is the K_M for catechol when tobacco extract was used it is apparent that the affinity of the mushroom enzyme for catechol is considerably greater than that of the tobacco enzyme. It seems apparent that more than one polyphenol oxidase is present in these species and that thorough purification must be accomplished before the individual components can be completely characterized.

It has been established that certain extracts from both tobacco roots and mushroom sporophores will catalyze the oxidation of dihydroxyphenylalanine, and caffeic acid, however, initial detailed studies of substrate specificities have been limited to chlorogenic acid and catechol. Preliminary experiments with crude extracts from potato show that catechol is about 60 % as effective as chlorogenic acid as a substrate for potato polyphenol oxidase(s). In this regard Yakushiji et al (9) have reported that chlorogenic acid was more rapidly oxidized in presence of potato polyphenol oxidase than was catechol.

SUMMARY

Evidence is presented indicating that crude tobacco extracts catalyze the oxidation of chlorogenic acid much more rapidly than catechol. Conversely the rates of oxidation of catechol catalyzed by mushroom extracts are much more rapid than those with chlorogenic acid. Evidence obtained from purification experiments indicate both mushroom and tobacco contain more than one enzyme capable of catalyzing the oxidation of polyphenolic compounds.

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ENDOGENOUS BUD AND ROOT FORMATION BY ISOLATED ROOTS OF CONVOLVULUS GROWN IN VITRO[1,2]

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Among the most successful weed species are numbered those which propagate themselves vegetatively by forming buds from underground tissues, especially root systems. Cultivation of the soil serves to break the root into numerous pieces, each capable of forming a new plant. Notable among the weeds of this type are members of the family Convolvulaceae, e.g., bindweed, *Convolvulus arvensis*, members of the Compositae such as the Canada thistle, *Cirsium arvense*, members of the Polygonaceae, e.g., sorrel, *Rumex acetosa* and many others. Bud formation by roots has been studied extensively from the morphological and anatomical standpoint, but little information is available concerning the physiological processes. In an extensive monograph on the subject, Beijerinck (1) described a large number of species in which bud formation by roots is the usual method of propagation. According to Priestley and Swingle (7) at least three types of bud formation by roots may occur. In the 1st type, bud formation is preceded by the formation of a callus tissue from cortical parenchyma or from deeper-lying tissues of the root and from this callus tissue mass are differentiated bud primordia. In the 2nd type, bud primordia develop from wound callus tissue which proliferates from the cut ends or damaged surfaces of the roots. Such buds may arise exogenously or have endogenous origin. The 3rd type of bud formation by roots involves the initiation of bud primordia within the deep-seated tissues of the root, i.e., from the pericycle. Such endogenous bud formation is closely comparable to endogenous lateral root initiation, occurring in pericycle cells usually located at the protoxylem points of the vascular cylinder.

Because of the importance of bud formation by root systems in the general problem of weed control and because of the fundamental problems involved in the determination of differentiating meristems, it was considered desirable to establish isolated root systems in culture as clonal materials to be used in studying the problem under controlled conditions.

Bud formation by isolated roots grown in vitro has been reported occasionally in the literature. White (14) illustrated bud formation from a callus formed on an isolated root segment of dandelion. This type of bud formation represents the 2nd type described above. Norton and Boll (6) also reported briefly on an isolated root system of a hybrid tomato which occasionally formed buds, again apparently after formation of a callus by the root. Bud formation by isolated root callus tissue grown in vitro is well known (5). Recently, Seeliger (8) illustrated cultured roots of *Robinia pseudoacacia* L., a woody species, whose roots produce endogenous buds in vitro.

In the present report is described the establishment in continuous culture of isolated roots of the common bindweed, *Convolvulus arvensis* L., which are capable of forming true endogenous buds in culture.

MATERIALS AND METHODS

Seed material was collected from the field in the late summer of 1952 in Northern California from the wild species, *Convolvulus arvensis* L. Seeds were surface sterilized by soaking for 15 minutes in a commercial preparation of sodium hypochlorite diluted to a final concentration of approximately 0.5 % by weight. Seeds were transferred aseptically to moistened sterile filter paper in Petri dishes and were allowed to germinate in the dark at 25° C for three days. At this time, 5-mm root tips were excised and transferred, one tip per flask, to 50 ml of liquid nutrient medium in 125-ml Erlenmeyer flasks. The flasks were placed in the dark at 25° C. Of the large percentage of roots which grew, one root showing particularly robust growth was selected for clonal propagation. All of the studies subsequently have been made with the same root clone which was first established in November 1952. The clonal material has been maintained in the dark, except for brief periods of fluorescent illumination during transfer, with continuous subculture approximately every four months. In certain experiments, roots in flasks were grown under fluorescent lights at approximately 100 and 200 ft-c at 25° C.

The medium used throughout these studies was based on the modified Bonner medium developed for the growth of pea roots (11). The medium contains the following constituents: 242 mg $Ca(NO_3)_2 \cdot 4\,H_2O$; 42 mg $MgSO_4 \cdot 7\,H_2O$; 85 mg KNO_3; 61 mg KCl; 20 mg KH_2PO_4; 1.5 mg $FeCl_3$; 40 g sucrose made up to one liter with glass distilled water. Usually the vitamins thiamin HCl at 0.1 mg/l and nicotinic acid at 0.5 mg/l were also added. In addition, trace elements were added as follows: 1.5 mg $ZnSO_4$; 4.5 mg $MnSO_4$; 0.25 mg $Na_2MoO_4 \cdot 2\,H_2O$; 1.5 mg H_3BO_3 and 0.04 mg $CuSO_4 \cdot 5\,H_2O$ per liter of solution. The pH of the medium was adjusted to give a final pH of 5.0 after sterilization. The complete medium was sterilized by autoclaving at 15 lbs/in.^{2} for 20 minutes. Flasks were capped over gauze-covered cotton plugs with unwaxed paper cups during the culture periods to decrease the rate of evaporation and protect against dust.

[1] Received March 1, 1958.

[2] This investigation was supported in part by grant number RG 2861 from the National Institutes of Health, Public Health Service. The author is indebted to Mr. Victor Duran for his photographic work.

Experimental Results

Establishment of the Clone and the Morphology of the Root in Culture: Isolated roots of Convolvulus grown in the medium described above show a marked apical dominance, which expresses itself in two ways—by the rapid elongation of the main axis tip and by an almost complete suppression of lateral root formation. Thus an isolated root tip excised from the radicle of a germinating seed elongates very rapidly. In the root tip used to establish the clone, the average elongation rate was 24 mm per day for the 1st three weeks. Upon transfer to fresh medium, the excised 10-mm tip continued to elongate at an average rate of about 25 mm per day. Lateral root formation was relatively infrequent. Such primary tips have been carried without transfer for up to 60 days in the same flask without any marked diminution in the elongation rate.

Establishment of a clone, however, must not depend upon propagation of the main radicle tip, but upon development of roots from segments cut from the radicle. It is with such segments that the experiments to be described are concerned. In establishing the clone, an excised primary root tip which had grown to a length of 480 mm by the 3rd week was used. The root was cut into 10-mm segments and each segment was transferred to fresh medium. In all experiments segments were chosen which did not possess macroscopically visible primordia, although the presence of either lateral roots or buds on the initial segment did not significantly change the course of development.

It is convenient to describe the sequence of events during the development of a root segment after its transfer to fresh medium continued in the dark. By the end of the 2nd week usually one lateral root has developed from the distal half of the root segment (i.e., distal with respect to the root from which it was derived). Occasionally a 2nd lateral root may be initiated, but it seldom elongates. The 1st lateral root to develop elongates slowly at first, then more rapidly, increasing in diameter until it achieves approximately the diameter of the segment from which it came; this diameter is maintained for the remainder of its elongation before sub-culture. The rate of elongation of lateral roots in the dark is lower than that for primary root tips, averaging between 10 and 20 mm per day over prolonged culture periods once the lateral root begins elongation. Usually concurrent with the initiation of the lateral root which will become the main root axis of the new culture, there are initiated on the inoculum segment one or more buds which at one week are just evident as swellings along the root segment and at two weeks are clearly evident morphologically as buds. By three weeks the buds may enlarge to 3 to 5 mm. Their distribution along the root segment is not fixed; they may occur near the lateral root or at the other end of the segment. Usually they occur proximal to the site of lateral root initiation (fig 1). Thus each root segment shows distinct polarity and becomes, in fact, a complete plant and no longer can be considered in the same category as isolated root segments, such as those of tomato, which in culture normally form only more root structure. Under these conditions in the dark, the bud on the initial root segment seldom elongates beyond 5 to 10 mm (table I). After the lateral root segment has reached the length of 500 mm or longer, endogenous buds may also be formed on the basal portions of the lateral root. In fig 2 is illustrated in flask culture a root started from a 10-mm segment after growing 20 weeks in the dark. In figure 3 is shown the extent of such a root after 20 weeks without transfer.

All of the buds formed by the root under these conditions are true endogenous buds, originating deep within the tissues of the root in the pericycle, usually opposite the protoxylem points of the primary vascular tissues of the main root axis (fig 4).

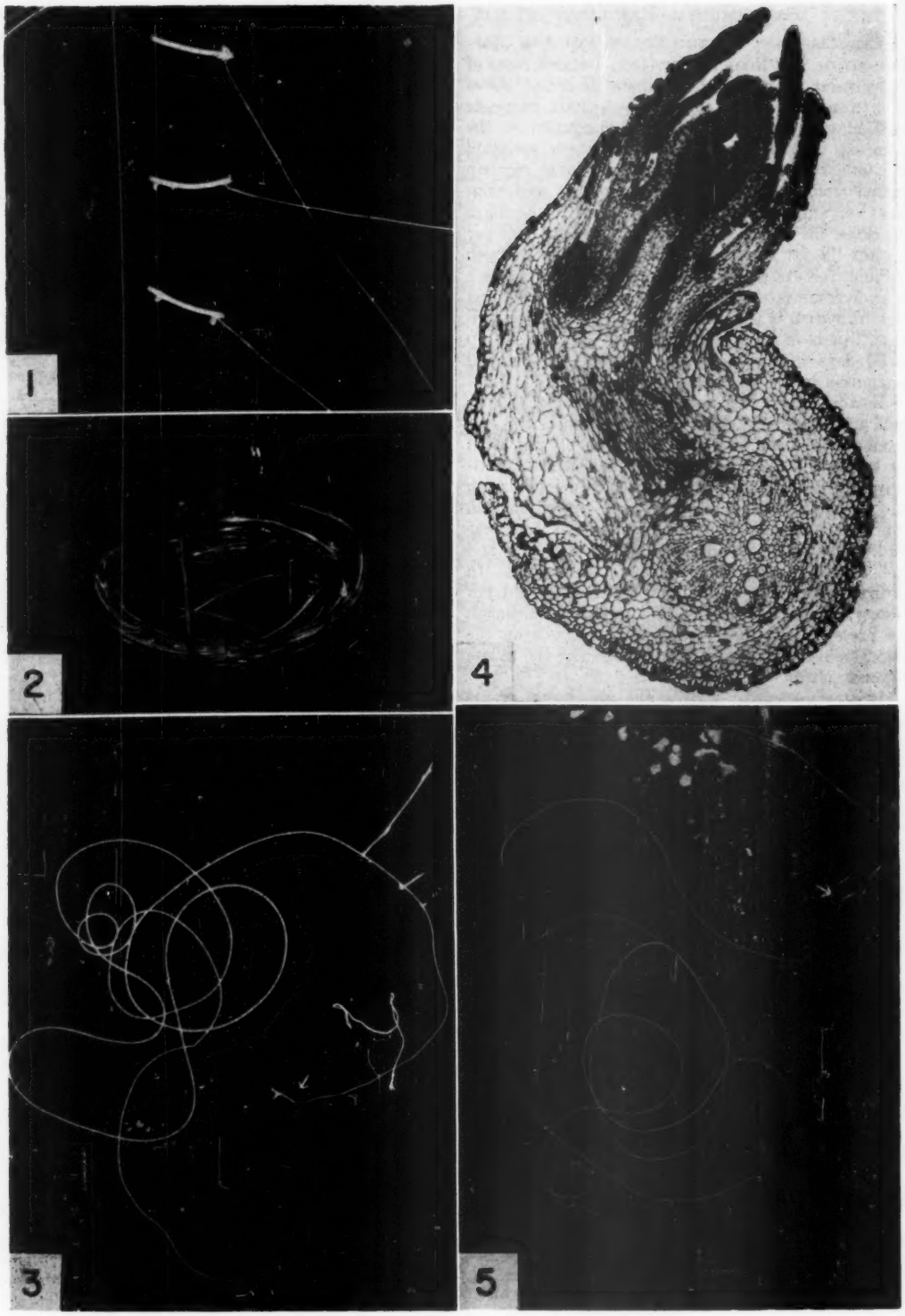
Development of 10-mm Root Segments in the Dark and in the Light: In table I are summarized the results of two experiments in which 10-mm root segments were grown in the dark on the control medium. These data illustrate quantitatively the se-

Table I

Development of Lateral Roots and Endogenous Buds by Convolvulus Root Segments Grown in Control Medium in the Dark and in the Light at 25° C *

	Second week		Fourth week		Seventh week		Fourteenth week	
	Dark	Light **	Dark	Light	Dark	Light	Dark	Light
Average number of lateral roots per segment	0.3	0.4	0.9	1.0	1.2	1.0	1.2	1.4
Average length of laterals per root in mm	1	5	35	128	244	181	1128	1207
Average number of buds per segment	0	0	0.5	1.0	2.0	1.0	2.0	1.0
Average bud length in mm	...	...	4	10	5	92	5	270

* Each figure is based on the average of 2 experiments each composed of 8 to 12 root segments.
** Illumination was about 100 ft-c from warm white fluorescent tubes on a 12-hr light-dark cycle.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2
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TABLE II

EFFECT OF KINETIN ON LATERAL ROOT AND BUD DEVELOPMENT IN CONVOLVULUS ROOT SEGMENTS GROWN IN THE DARK AND LIGHT AT 25° C *

CONCENTRATION OF KINETIN (MG/L)		AV NO. OF LATS/SEGT		AV LENGTH OF LATS (MM)		AV NO. OF BUDS/SEGT		% OF SEGTS WITH TERMINAL BUDS	
		WEEK		WEEK		WEEK		WEEK	
		7	20	7	20	7	20	7	20
0	Dark	1.3	1.4	198	1025	1.5	2.8	0	0
0	Light **	1.1	1.3	116	160	1.6	1.7	0	0
0.01	Dark	1.0	1.5	218	1252	0.6	2.2	0	11
0.01	Light	1.0	1.0	111	400	1.1	1.4	50	72
0.1	Dark	1.3	1.4	297	1591	0.1	2.2	11	50
0.1	Light	0.7	0.7	20	21	1.4	3.0	90	100

* Each figure is based on the average of 12 to 15 root segments.
** Illumination was about 200 ft-c from mixed warm white and daylight fluorescent tubes on a 12-hr light-dark cycle.

quence of events described above. It should be noted that lateral roots become apparent first and bud primordia develop shortly thereafter.

A parallel series of root segments grown in the control medium was illuminated with mixed warm-white and daylight fluorescent lights at approximately 100 ft-c. From a study of the data in table I it becomes apparent that, at these low light intensities, root development is similar in most respects to that in dark-grown roots. Whether in the light or dark, each segment usually produces only one lateral root and one or two buds. Lateral root elongation, while initially slightly accelerated in the light, later is much the same under the two conditions. The major difference in behavior is evident in the response of the buds, which elongate in the light, forming many leaves while buds in the dark usually are completely inhibited or elongate only slightly and develop no expanded leaves. In figure 5 is illustrated a plant after 20 weeks in alternating 12-hour periods of dark and light, showing bud elongation and numerous leaves. At somewhat higher light intensities (see table II), it has been found that lateral root elongation can be markedly inhibited, as has been observed in cultured pea roots (10). Comparison of figure 3 with figure 5 shows that roots grown in continuous dark achieve a larger root diameter than roots exposed to periods of light.

It is difficult from the experiments carried out to date to sort out any consistent correlation phenomena among bud and root initiation or elongation other than the fact that lateral root initiation precedes bud initiation under these conditions of culture and that organ formation by the root segments appears to be rather specifically limited.

EFFECTS OF GROWTH FACTORS ON BUD FORMATION IN THE LIGHT AND DARK: A number of experiments have been performed in which specific growth factors which might affect organ formation in these root segments have been added separately or together to the control medium. Indoleacetic acid (IAA) in concentrations between 10^{-8} M and 10^{-12} M was found to have essentially no effect on organ formation in roots grown in the dark. IAA at 10^{-6} M, while having no effect on root segments cultured in the light, produced extensive swelling and disruption of the cortical tissues at both ends of the root segments in the dark. At this concentration, all organ formation was suppressed by IAA in dark-grown roots. Adenine sulfate, when tested on roots grown in the dark over a range of concentrations between 0.4 and 40 mg/l, was found to have no evident effect on organ initi-

FIG. 1. Root segments of Convolvulus grown on agar medium in the dark for 7 weeks, each showing the development of a lateral root and one or more endogenous buds. ×1.2.

FIG. 2. Ten-mm root segment of Convolvulus grown in liquid medium in the dark for 20 weeks. Total length of lateral root was about 1600 mm. Note several elongate etiolated buds produced along the length of the lateral root. ×0.8.

FIG. 3. Root segment of Convolvulus grown in liquid medium in the dark for 20 weeks, showing the extent of root development. Original 10-mm segment (indicated by arrow) formed 2 lateral roots and 2 buds. Total length of lateral roots was 1130 mm; 5 buds were formed by the lateral root. ×0.6.

FIG. 4. Transverse section of root segment of Convolvulus grown in liquid medium in the dark for 20 weeks, showing the endogenous origin fom the pericycle opposite one of the primary xylem points, of a bud which has been cut longitudinally. ×70.

FIG. 5. Root segment of Convolvulus in liquid medium in alternating 12-hr periods of dark and light of 100 ft-c from white fluorescent tubes. Note the development of the shoot system from the original 10-mm segment (indicated by arrow). Compare the diameter of the lateral root with that in fig. 3. ×0.6.

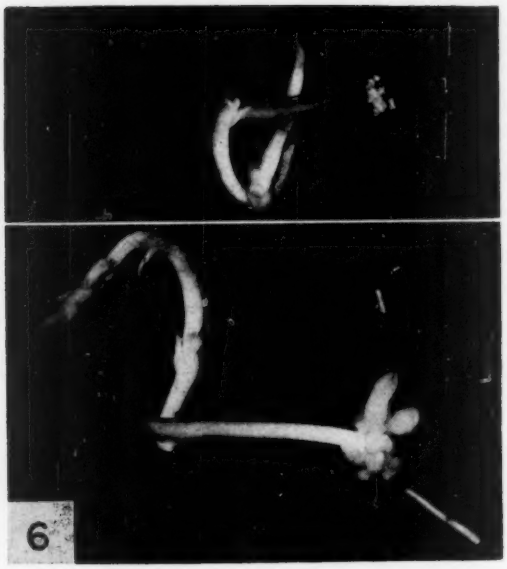

FIG. 6. Bud formation of segments of Convolvulus root grown for 8 weeks in liquid medium containing kinetin under alternating 12-hr periods of dark and 100 ft-c white fluorescent light. Top, 0.01 mg/l kinetin; bottom, 0.1 mg/l kinetin. ×3.5.

ation. The following substances, tested because of their physiological activity in a variety of plant tissue systems, were also found to have no effect on organ initiation at the concentrations tested: Difco Bacto yeast extract, casein hydrolysate (enzymatic), pyridoxine HCl, calcium pantothenate, choline HCl, folic acid, *p*-aminobenzoic acid, inositol, riboflavin, biotin, glycine, and 2,4-dichlorophenoxyacetic acid.

A positive controlling influence on organ initiation was found when kinetin (6-furfurylaminopurine) was tested. In table II are summarized the results of a typical experiment in which kinetin was added to the control medium at 0.01 and 0.1 mg/l and the roots were grown both in continuous darkness and in 12-hour periods of alternating dark and light at 200 ft-c. In the medium lacking kinetin, development was similar to that described in table I, except that at the higher light intensity lateral root elongation was markedly inhibited. This inhibition of root elongation was accentuated by the presence of 0.1 mg/l kinetin, which in the dark appears to stimulate root elongation. Root segments grown in the presence of 1.0 mg/l kinetin turned brown, whether in the light or dark, due apparently to toxicity.

At the concentrations shown in table I, kinetin does not significantly affect the number of lateral roots initiated either in the light or the dark. In dark-grown root segments, kinetin slows the development of buds but appears not to affect significantly the number of buds ultimately formed by the segments.

By far the most striking effect of kinetin on these root segments is a stimulus to the formation of bud primordia at the distal end of each root segment. These structures, which appear to be incompletely organized bud primordia, occur only at the cut surface of the root-end of the root segment. Their size and number are dependent on the concentration of kinetin and whether the root is grown in the light or the dark. In figure 6 are illustrated root segments grown for 8 weeks in the light in the presence of two concentrations of kinetin. At 0.01 mg/l kinetin, the distal end of the root segments appear swollen as if with a callus formation, but distinct structures are evident. At the higher concentration of kinetin, the bud primordia are larger in size and more numerous. An accurate count of these structures is difficult to make except on histological preparations. Some indication of the effectiveness of kinetin in the formation of these structures is given in table II by the percentage of segments which show this characteristic response to kinetin treatment. The greatest response occurs in roots cultured in the light, but with time a proportion of segments grown in the dark also responds. Careful histological analyses of these structures will be necessary before any relationship between them and normal endogenous bud formation can be established.

DISCUSSION

The capacity for endogenous bud formation is a distinctive character of a relatively few groups of plants and involves a mechanism about which we have very little physiological knowledge. In the present experiments, the inherent capacity of Convolvulus roots to form endogenous buds has been retained by isolated roots through successive sub-cultures for several years apparently without signs of diminution. Thus, under these conditions of culture, the root itself is capable of providing the metabolites and cell division factors which are essential for the initiation, organization and partial development of the buds. Light is required for the complete development of the shoot system. It is probable that bud initiation, like lateral root initiation (12), is dependent upon a number of specific chemical factors formed by the root in culture. The present experiments suggest that some factor of the kinin-type may be involved in bud initiation by Convolvulus roots. It is interesting that kinetin is much more active when roots are grown in the light than in the dark. This result suggests the possibility of an interaction of factors such as has been reported by Skoog and Miller (9) in tobacco pith tissue.

Danckwardt-Lillieström (2) has recently shown that isolated roots of *Isatis tinctoria*, which in first passage normally regenerate shoots from callus formed at the cut surface, lose this capacity on repeated sub-culture. If kinetin is supplied to roots in these later passages, shoot initiation takes place. In this case, it would seem that the root is depleted of a

kinetin-like factor during subculture which must then be replenished via the nutrient medium.

In studies with the thickened roots of Taraxacum and Cichorium, Warmke and Warmke (13) reported that auxin distribution within root cuttings seemed to control the capacity of the root tissues for organ formation; roots were initiated at the morphologically distal end of the root segments which were relatively high in auxin content, whereas treatment which reduced the total auxin content of the segments resulted in leaf initiation from the distal ends in a few cases. Emery (4) found that IAA treatment of root cuttings of the fireweed, *Chamaenerion angustifolium*, resulted in the initiation of large numbers of lateral roots, but inhibited bud initiation. Other studies with thickened root segments show a similar inverse relation between auxin level and bud initiation. Whether kinetin-like substances interact in these systems has not been studied. In the root segments of Convolvulus studied here, no simple interaction of kinetin with auxin was observed over the range of concentrations tested.

It will be of both theoretical and practical interest if, using such systems as described here, one can both stimulate and inhibit bud initiation by chemical treatment or convert potential lateral root primordia to bud primordia or vice versa. It remains to be investigated whether at some stage in Convolvulus an "indifferent" meristem is formed as has been reported, for example, in roots of horseradish (recently reviewed by Dore (3)). Such meristems can become either root or shoot meristems, depending upon the physical-chemical environment at the time of initial development. The situation described here offers an ideal system in which to study the physiology of these and related processes.

Summary

Excised roots of the common bindweed, *Convolvulus arvensis*, were cultured in a sterile synthetic nutrient medium containing the usual macro- and micronutrient elements, sucrose, and the vitamins thiamin and nicotinic acid. A clone of roots has been maintained in continuous culture in this medium with regular transfers approximately every four months for over five years. Ten-mm root segments grown in liquid medium in the dark usually produce a single lateral root which becomes the main root axis of the new culture. The lateral root develops slowly at first and then achieves an elongation rate of up to 20 mm per day. The initial root segments also typically form one or two endogenous buds which originate in the pericycle opposite the primary xylem poles in the same anatomical position that lateral roots originate. Low intensity white fluorescent light (100 ft-c) has relatively little effect on root elongation, but stimulates shoot development which does not proceed in the dark. At higher light intensities, root elongation is inhibited. Of a large number of growth factors tested by adding to the nutrient medium, only kinetin (6-furfurylaminopurine) influenced organ initiation by Convolvulus root segments. In the presence of 0.1 mg/l kinetin, root segments grown in the light produced a large number of bud primordia at the cut distal end (root end) of the segment. In the dark the stimulation to bud initiation by kinetin was much less marked. It is concluded that externally supplied kinetin induces bud initiation in cultured Convolvulus root segments and that light markedly augments this induction.

The author expresses his appreciation to Dr. H. B. Currier for provision of seeds of Convolvulus and to Dr. J. B. Wright of the Upjohn Company for a supply of kinetin.

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THE OSMOTIC CELL, SOLUTE DIFFUSIBILITY, AND THE PLANT WATER ECONOMY [1]

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The question which prompts the present work is: "How can the concept of the plant cell as an osmometer with semi-permeable walls be justified, when solutes may enter the plant cell vacuole?" Textbooks of plant physiology and physical chemistry treat osmosis in detail only for equilibrium conditions and non-diffusible solutes. To extend the quantitative treatment to the case of a diffusible solute we must abandon the equilibrium approach and study the problem dynamically. We introduce the dynamic approach by applying it to the classical cell osmometer; then we proceed to the case of a diffusible solute.

DYNAMICS OF THE CLASSICAL CELL OSMOMETER: We use the following symbols:

V, cell volume (cm^3). V_o, cell volume at zero turgor pressure.

$v = V/V_o - 1$, relative departure of cell volume from V_o.

A, external surface area of cell (cm^2). A_o, value of A at zero turgor pressure.

P, osmotic pressure of cell contents (in the sense of Meyer (14), i.e., as a concentration-dependent property of the solution, independent of the hydrostatic system) (atm).

T, turgor pressure (atm). [Broyer and others (4, 21, 22, 27) have shown the general equivalence of T and "wall pressure," and that it is a mistaken over-emphasis of an infinitesimal second-order effect to dwell on the distinction between them (6)].

K_w, permeability of cell wall to water (cm sec^{-1} atm^{-1}). t, time (sec).

We suppose that, initially, $T = 0$, and $P = P_o$, and that the solutes in the system are non-diffusible. If the cell is now placed in free water, water will enter the cell until the (increasing) turgor pressure becomes equal to the (decreasing) osmotic pressure. The dynamics of this process is described exactly by the equation.

$$dV/dt = K_wA[P - T] \tag{1}$$

subject to the initial condition

$$t = 0, V = V_o \tag{2}$$

Quite generally, K_w, A, P and T are functions of V. If these functions are known, equation 1 may be integrated. If experimental values of these functions are used, numerical integration will generally be necessary. This presents no great difficulty, but it is simpler to use certain approximations which enable the problem to be solved analytically.

A increases with V (in fact $A \approx A_o(1 + 2v/3)$ for isotropic swelling and shrinking) and K_w may vary with V (e.g., (2)). This is unlikely to change the order of magnitude of K_wA, which affects the time scale of osmometer dynamics, though not its general character. We therefore simplify the analysis by taking K_wA as constant and equal to K_wA_o.

If we take P proportional to solute concentration (as it is to a first approximation)

$$P = P_oV_o/V = P_o/(1 + v) \tag{3}$$

If we assume that change of cell volume is proportional to change in turgor pressure, we have for the T(V) function

$$T = (V/V_o - 1). \text{ i.e. } T = \epsilon v \tag{4}$$

The elastic modulus, ϵ, corresponds to the "coefficient of enlargement" of Broyer (5). As Broyer states, ϵ is not strictly constant for perfectly elastic cell walls, except for infinitesimal volume changes. Since the assumption that the wall obeys Hooke's law is, in any case, an approximation (9), it seems permissible to adopt ϵ as constant in the present analysis.

We recognize that, in real plants, marked deviations from the linear T(v) relation to be used here may occur. In this sense the present work may be considered a first approximation. Essentially similar results would follow from a more precise, though more elaborate analysis along the same lines in which non-linearity in T(v) was taken into account.

The existence of a unique T(v) function implies, however, that the cell volume changes are elastic. Obviously, the plastic, irreversible, deformations associated with cell elongation are beyond the scope of the present treatment, though it may prove possible to include these in a similar, but more complicated, analysis. Insofar as our special concern here will be with cells at low levels of turgor, this is not a serious limitation.

Using equations 3 and 4 in equation 1 then gives

$$\frac{dv}{dt} = \frac{K_wA_o}{V_o}\left[\frac{P_o}{(1 + v)} - \epsilon v\right] \tag{5}$$

This is integrable, but the further developments will be simplified if we use the fact that v is generally rather smaller than unity and introduce the approximation

$$P_o/(1 + v) = P_o(1 - v) \tag{6}$$

[1] Received February 27, 1958.

[2] Address for 1958. This work was supported in part by a grant from the Rockefeller Foundation, RF 56160.

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Equation 5 may then be rewritten

$$\frac{dv}{dt} = \frac{K_w A_o}{V_o}[P_o - (\epsilon + P_o)v] \qquad (7)$$

The particular integral of equation 7 which vanishes for $t = 0$ (in accord with equation 2) is

$$v = \frac{P_o}{(\epsilon + P_o)}\left[1 - \exp\left(-\frac{(\epsilon + P_o)K_w A_o}{V_o}t\right)\right] \qquad (8)$$

Figure 1 shows the approach of the ideal osmometer to the new equilibrium, $v = P_o/(\epsilon + P_o)$, according to equation 8. The predicted behavior is quite similar to the observed behavior of plant tissues (e.g., (7, 16, 23)), though certain inherent differences are to be expected between the behavior of an individual cell and of a tissue which is an aggregation of cells.

The half-time of the approach to the new equilibrium is $\frac{0.693V_o}{A_oK_w(\epsilon + P_o)}$. That is, for a given cell dimensions and permeability, the speed of adjustment is directly proportional to $(\epsilon + P_o)$. Thus both the elastic properties of the cell wall and the quantity of solute in the cell exert important influences on cell dynamics.

It will be noted that the relationships used here fail for the plasmolyzed cell, and that the analysis is restricted to cells in the normal unplasmolyzed condition.

The preceeding treatment may be applied to more general problems in the dynamics of the classical cell osmometer. No now principles are involved in such generalizations.

"Osmosis" in the Presence of a Diffusible Solute: The propriety of the term "osmosis" is open to question if the solute is diffusible. Strictly, the process is one of dialysis. Just as in the ideal osmometer, a diffusion pressure (14, 1) difference is set up across the membrane. In the ideal case, net movement of solvent across the membrane may be prevented by applying a constant hydrostatic pressure difference. In the present case, however, "osmotic" equilibrium would require that the applied pressure difference vary in accord with changes in concentration produced by diffusion of the solute.

The question arises as to whether the "osmotic pressure" depends only on the concentrations of solute or whether it is influenced by the permeability of the membrane to the solute. An argument similar to that given by Ostwald (quoted in (10)) to demonstrate the equality of the osmotic pressures developed by all ideal membranes shows that the transient osmotic pressures we examine here also depend only on the solute concentrations. The viewpoint of Hildebrand (12) that osmosis "is primarily a consequence of the tendency of two different liquid species, under the impulse of thermal agitation, to achieve a state of maximum disorder by any available path, and that the route via osmosis is no more significant theoretically than one via the vapor state" leads to the same conclusion.

Cell Dynamics in the Presence of a Diffusible Solute: After the cell we have considered above attains its final equilibrium, let it be removed and immersed in a large body of a solution of diffusible solute. In considering subsequent cell behavior, we use the following additional symbols:

P_n, osmotic pressure of the cell contents due to non-diffusible solutes (atm).

P_d, osmotic pressure of the cell contents due to diffusible solute (atm).

P_1, osmotic pressure of the external solution (of diffusible solute) (atm).

K_s, apparent permeability of cell wall to diffusible solute, essentially defined by equation 10 (cm sec^{-1}).

c_1, concentration of diffusible solute in the external solution (g cm^{-3}).

c, concentration of diffusible solute in the cell contents (g cm^{-3}).

q, quantity of diffusible solute in the cell (= cV) (g).

Our model of solute movement consists of purely passive diffusion. It is for this reason that we call K_s apparent permeability. The simple proportionality between rate of solute movement and concentration difference implicit in the model is certainly not always realized in plant cells. Nevertheless the model enables some exploration of the effects of solute diffusibility on osmotic behavior of the cell.

The system of equations describing the movement of water and diffusible solute is then

$$dV/dt = K_w A(P_n + P_d - P_1 - T) \qquad (9)$$

$$dq/dt = K_s A(c_1 - c) \qquad (10)$$

Equations 4 and 6 enable us to rewrite equation 9 as

$$\frac{dv}{dt} = \frac{K_w A_o}{V_o}[P_o + P_d - P_1 - (\epsilon + P_o)v] \qquad (11)$$

We must establish P_d as a function of time before we can integrate equation 11. All volume changes are relatively small, so we may introduce the approximation that, even though K_s, A and V all vary, the quantity K_sA/V is constant and equal to K_sA_o/V_o. This enables equation 10 to be reduced to

$$\frac{dc}{dt} = \frac{K_s A_o}{V_o}(c_1 - c) \qquad (12)$$

Then, for P_1, P_d proportional to c_1, c (as they will be to a first approximation) equation 12 becomes

$$\frac{dP_d}{dt} = \frac{K_s A_o}{V_o}(P_1 - P_d) \qquad (13)$$

Now $P_d = 0$ at $t = 0$, so P_d is given by the particular integral of equation 13,

$$P_d = P_1\left[1 - \exp\left(-\frac{K_s A_o}{V_o}t\right)\right] \qquad (14)$$

Using equation 14 in equation 11, we obtain

$$\frac{dv}{dt} = \frac{K_w A}{V_0}\left[P_o - P_1' \exp\left(-\frac{K_s A_o}{V_o} t\right) - (\epsilon + P_o)v\right], \quad (15)$$

which is subject to the initial condition

$$t = 0,\ v = P_o/(\epsilon + P_o) \quad (16)$$

Numerical solutions of equation 15 subject to condition 16 are shown in figure 2. The most important quantity governing the time-dependence of the cell-behavior is $K_s A_o/V_o$. For this reason the curves are plotted against the dimensionless quantity $K_s A_o t/V_o$. The other significant parameter of the system, $(\epsilon + P_o)K_w/K_s$, influences the relative rate of approach to the initial quasi-equilibrium.

An idea of orders of magnitude can be gained by inserting numerical values. $A_o/V_o = 10^3$ for a spherical cell of radius 3×10^{-3} cm. K_s is difficult to evaluate, but may be about 5×10^{-8} for plant cell membranes and the salts of interest here (19, 8). The experimental data on K_w indicates 5×10^{-7} as typical for plant cells (11). Broyer (3) has deduced $\epsilon \approx 60$ for potato tuber tissue while Ordin and Bonner's data for Avena coleoptiles (16) yield $(\epsilon + P_o) \approx 100$. We may therefore take $(\epsilon + P_o)$ of order of magnitude 50 to 100. On the basis of these values, the unit of dimensionless time scale of figure 2 would be about 6 hours, while $(\epsilon + P_o)\ K_w/K_s$ would be of order 500 to 1000. The curves of figure 2 cover the range 50 to 5000.

It is seen that the behavior of the cell on immersion in a solution of diffusible solute has three phases: (i) a very rapid adjustment to V and T values little different from those which would be developed by the ideal osmometer; (ii) a period of quasi-equilibrium, during which the classical equilibrium values of V and T continue to be approximately realized; (iii) a slow drift back to the initial condition of cell volume and turgor.

It must not be supposed, however, that the cell has returned to its initial state. Whereas the osmotic pressure of the cell contents was initially $P_o\epsilon/(\epsilon + P_o)$, it has now increased to $P_o\epsilon/(\epsilon + P_o) + P_1$.

Accordingly, if the cell is now removed from the solution of diffusible solute and replaced in free water, it will increase in volume rapidly until v approaches the value $P_o/(\epsilon + P_o) + P_1/\epsilon$ (i.e., T approaches the value $P_o\epsilon/(\epsilon + P_o) + P_1$). This will be followed by a gradual (nearly exponential) return to a state in which $v = P_o/(\epsilon + P_o)$. If, after this state is attained, the cell is removed from the free water and placed in a solution of non-diffusible solute of osmotic pressure P_o, the volume decreases rapidly to V_o, the turgor pressure to zero and the osmotic pressure of the cell contents to P_o. The cell has now returned to the state in which it was originally introduced. Figure 3 presents schematically the cycle of operations to which we have subjected it. The osmotic pressure history of both external solution and cell contents, and the volume (or turgor) changes which the cell undergoes, are shown.

Cell Dynamics with Changing External Concentration of Diffusible Solute: It will be clear from the preceding section that deviations from classical osmotic behavior induced by the presence of diffusible solutes become apparent only some time after external conditions change. We now consider cell behavior when the external concentration of diffusible solute changes gradually but continuously, as one might expect in nature.

Let us interrupt the previous cycle of operations on the cell at the point where it has reached equilibrium with free water (C of fig 3). We now suppose that a diffusible solute is introduced continuously into the water, so that, at any time, t, the osmotic pressure of the external solution is αt.

The equation governing the increase of osmotic pressure of the cell contents due to the entry of diffusible solute is again equation 13. P_1 is not constant here, but is equal to αt, so that we have

$$\frac{dP_d}{dt} = \frac{K_s A_o}{V_o}(\alpha t - P_d) \quad (17)$$

The particular integral of equation 17 which vanishes for $t = 0$ is:

$$P_d = \alpha t - \frac{\alpha V_o}{K_s A_o}\left[1 - \exp\left(-\frac{K_s A_o}{V_o} t\right)\right] \quad (18)$$

Equation 11, describing the volume change of the cell, holds here also. However, $(P_d - P_1)$ is now

$$-\frac{\alpha V_o}{K_s A_o}\left[1 - \exp\left(-\frac{K_s A_o}{V_o} t\right)\right],$$

so that the equation becomes

$$\frac{dv}{dt} = \frac{K_w A_o}{V_o}\left[P_o - \frac{\alpha V_o}{K_s A_o}\left[1 - \exp\left(-\frac{K_s A_o}{V_o} t\right)\right] - (\epsilon + P_o)v\right] \quad (19)$$

subject to the condition

$$t = 0,\ v = P_o/(\epsilon + P_o) \quad (20)$$

Provided $(\epsilon + P_o)\ K_w/K_s$ is much greater than unity (we have estimated the probable range of values at 50 to 5,000), the solution of equation 19 subject to 20 is, to a high degree of accuracy

$$v = \frac{P_o}{\epsilon + P_o} - \frac{\alpha V_o}{(\epsilon + P_o)K_s A_o}\left[1 - \exp\left(-\frac{K_s A_o}{V_o} t\right)\right] \quad (21)$$

This result is shown graphically in figure 4. It will be seen that, no matter how high the external concentration of diffusible solute becomes, the consequent change in v cannot exceed $\alpha V_o/(\epsilon + P_o)K_s A_o$. Thus,

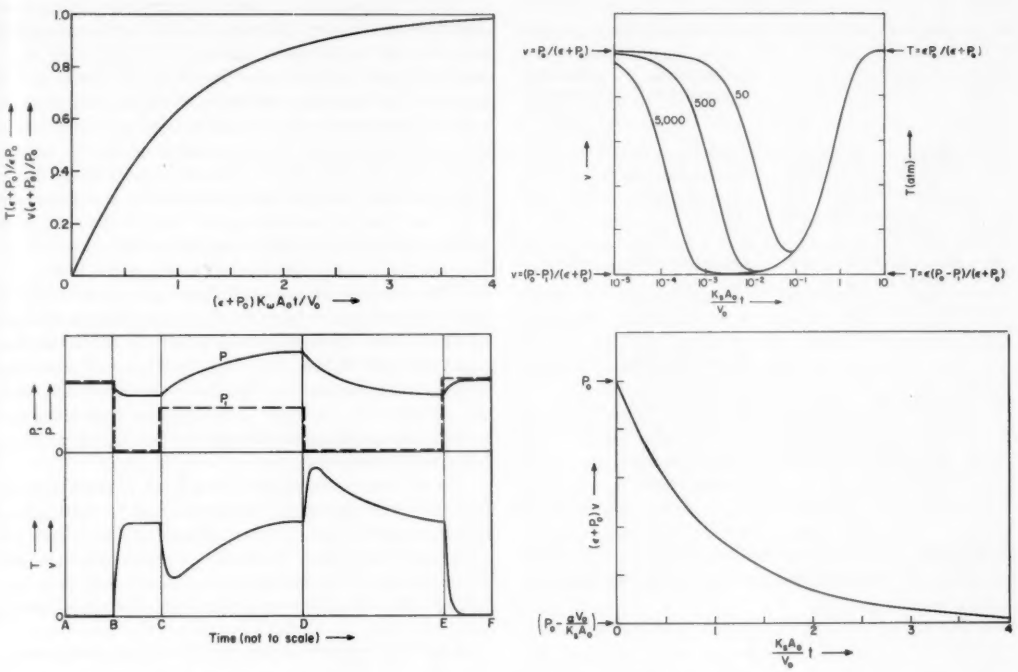


FIG. 1 (*top left*). The dynamics of the classical osmometer. Transition from zero to full turgor on immersion in free water.

FIG. 2 (*top right*). Cell behavior on immersion in solution of diffusible solute. The numbers on the curves denote values of $(\epsilon+P_0)K_w/K_s$.

FIG. 3 (*bottom left*). Schematic diagram of cycle of operations to which cell is subjected. Upper full curve represents osmotic pressure of the cell contents, P. Broken curve represents osmotic pressure of the external solution, P_1. Points in the time sequence:

A. Cell at zero turgor in equilibrium with non-diffusible external solution of osmotic pressure P_0.
B. Cell placed in free water.
C. Cell removed and immersed in solution of diffusible solute.
D. Cell returned to free water.
E. Cell returned to original non-diffusible solution.
F. Cell at zero turgor in equilibrium with original non-diffusible solution.

FIG. 4 (*bottom right*). Cell behavior with changing external concentration of diffusible solute.

if we assume a change in external osmotic pressure (due to diffusible solute) of 1 atm per day ($\alpha = 1.16 \times 10^{-5}$) and takes $K_s A_0/V_0 = 5 \times 10^{-5}$, $(\epsilon + P_0) = 75$, the maximum change in v is 0.0032. That is, the maximum change in cell volume is less than 1 in 300.

The behavior of cell volume, and the osmotic pressure of external solution and cell sap, is shown in figure 5 for this numerical example ($P_0 = 15$). The external osmotic pressure is allowed to increase 1 atm per day for 20 days, after which it remains constant. Note that the slight loss of turgor is quickly regained once the external concentration ceases to increase.

CELL DYNAMICS AND DONNAN PHENOMENA: To this point we have referred to "diffusible" and "non-diffusible" solutes without specifying whether the dissolved material is ionic or molecular. Only where the Donnan phenomenon operates need this be done. Table I summarizes various combinations of constitution of cell contents and external solution, and indicates the presence or absence of the Donnan phenomenon in each case.

Case 1 occurs, for example, during plasmolytic determination of osmotic pressure. Case 2 arises when the degree of ionization of the cell contents is negligible, and is perhaps improbable. Case 3, the most general one, is relevant, for example, to the interaction of a plant with the soluble salts of the soil.

The effect of the Donnan phenomenon, where it operates, on the previous analysis may be gauged by considering the problem treated under "Cell Dynamics in the Presence of a Diffusible Solute." Let us suppose that certain diffusible ions, originally in the cell, remain bound there, due to the presence of non-diffusible ions of opposite charge, so long as there are

TABLE I

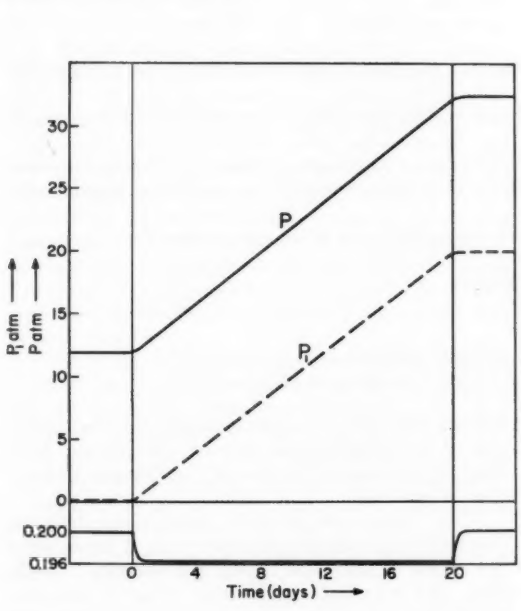
CASE	CELL CONTENTS	EXTERNAL SOLUTION	DOES DONNAN PHENOMENON OPERATE?
1	Diffusible electrolyte or non-electrolyte, plus (possibly) non-diffusible components	Non-diffusible electrolyte or non-electrolyte	No
2	Non-diffusible electrolyte or non-electrolyte, plus (possibly) diffusible non-electrolytes	Diffusible electrolyte or non-electrolyte, plus (possibly) non-diffusible components	No
3	Electrolyte with at least one diffusible ion, plus (possibly) non-diffusible components	Diffusible electrolyte, plus (possibly) non-diffusible components	Yes

no diffusible ions in the external solution. Let these "bound" ions contribute (at zero turgor pressure) a partial osmotic pressure P_1.

It is found that, if these ions are treated as non-diffusible while they remain bound, but as diffusible once the cell is placed in the large body of electrolyte solution, the analysis developed above predicts the final equilibrium osmotic pressure of the cell contents with a fractional error of about $P_1^2/8\ P_1(P_o + P_1)$. This is clearly small if P_1 is rather less than P_1 and P_o. Thus, for $P_o = 15$, $P_1 = 5$, $P_1 = 2$, the error is 1/200. The Donnan phenomenon produces a much greater effect on the concentrations of the individual ionic species than on the total concentration, which is the significant quantity influencing osmotic pressure.

The question of how the Donnan phenomenon affects the dynamics of approach to equilibrium is somewhat obscure. Diffusion rates of electrolytes (or their ions) through membranes may well be of the same order of magnitude whether Donnan effects are present or not. If so, the overall dynamic picture presented above remains relevant in the presence of the Donnan phenomenon.

OSMOMETER ANATOMY AND REAL PLANT CELLS: In this study the model we have used is the classical cell-osmometer, with the complication added that certain solutes are free to diffuse through the cell wall. The classical cell-osmometer wall is envisaged as providing: (i) the geometrical limits of the cell; (ii) the



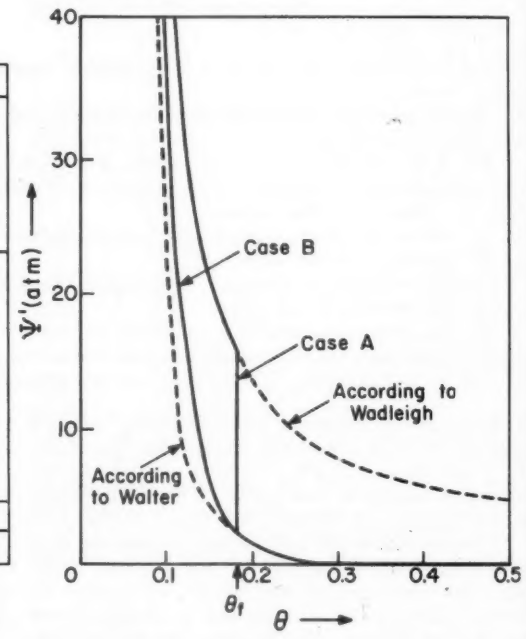


FIG. 5 (*left*). Cell behavior with changing external concentration of diffusible solute. Upper full curve represents osmotic pressure of the cell contents, P. Broken curve represents osmotic pressure of the external solution, P_1. Lower full curve shows relative cell volume, v.

FIG. 6 (*right*). Variation of "effective soil moisture stress," Ψ', with volumetric moisture content, θ, as implied by various theories. Upper broken curve—according to Wadleigh (25, 24). Lower broken curve—according to Walter (26). Solid curves—behavior indicated by present theory for cases A and B. θ_f denotes value of θ at failure of liquid continuity between soil water and root cell.

mechanical strength and elasticity supporting the turgor pressure; (iii) the semi-permeable membrane.

In the mature vacuolated plant cell, there are, as well as the wall and the vacuole, the plasmalemma, the cytoplasm and the tonoplast. Either of the membranes may operate as the effective resistance to the passage of water or of a particular solute; the cytoplasm itself may provide a significant resistance, or may have osmotic properties distinct from those of the vacuole.

A model sufficiently complex to include all these effects could be developed. However, the number of parameters in the system would be large, and the analysis cumbersome, even though the general results would not differ greatly from those we have obtained here for a simpler model. Nevertheless, there are points where some ambiguity arises when we apply the classical model to real plant cells.

Thus we have, throughout, referred to cell volume and cell contents, though we recognize that, where the effective barrier is the tonoplast, it would be more appropriate to work with vacuolar volume and vacuolar contents. The question of how cell volume varies with vacuolar volume raises such matters as change in cytoplasm volume, and the means of transmission of turgor from the cell wall across the cytoplasm to the vacuole. Further, we have referred all permeabilities to cell surface area, though, where the barrier between the external solution and the vacuole resides away from the cell wall, a smaller area (nevertheless, of the same order of magnitude) should, strictly, be used.

SOLUTE DIFFUSIBILITY AND PLANT WATER ECONOMY: In the real plant, both concentration gradients and effective diffusion cross-section per cell may be less than for the single cell, so that one can expect the effects of solute diffusibility to be somewhat retarded. However, with this qualification, the present analysis appears to be pertinent, and we conclude that solute diffusibility exerts a real influence on the water balance of whole plants.

Maximov (13) quotes work by a number of investigators which supports our analysis and this conclusion. Thus Renner added 1 % KNO_3 to water cultures of beans, and found that absorption of water was immediately reduced, but that subsequently absorption became more rapid, the plant apparently adapting itself to the change in solution. Monfort observed that a sudden increase in concentration of the external solution caused a temporary cessation of guttation; guttation was subsequently renewed, finally increasing above the original level. Ursprung and Blum found that immersion in concentrated solutions increased the suction pressure of the cells in the absorbing zone of the root. All these experiments follow the course predicted in figure 2, though it is possible that some other effect, such as increase in membrane permeability, operates where the final uptake rate is in excess of the initial one.

Also significant are the experiments of Rybin. After immersion of plant roots in salt solution had reduced water uptake to about half its normal value, the solution was suddenly replaced by pure water. There was an immediate increase in absorption to about 1.5 times the normal rate. On continued immersion in the water, the absorption rate approached its initial value. Sabinin observed similar phenomena. These experiments follow the course predicted in phases CDE of figure 3, if we regard the observed absorption rate as proportional to the difference in diffusion pressure deficit between the root cells and the solution.

The concept of "physiological dryness" (20) in saline soils has had a long currency in the literature of plant ecology. This viewpoint presupposes that the plant behaves as an ideal osmometer. The osmotic pressure of the soil water due to the soluble salts of the soil (which are diffusible through the plant membranes) is envisaged as opposing the entry of water into the plant via the root system. Wadleigh (25, 24) has used the "total soil moisture stress," defined as the sum of the tension and the osmotic pressure of the soil water, as a measure of the diffusion pressure deficit which must be exceeded in the epidermal cells of plant roots before water may be absorbed by them. Expressed thus, the concept is unexceptionable. However, the concept would seem to be of use only if the external electrolyte fails to diffuse into the cells of the root.

The analysis of solute diffusibility presented here indicates that the concept of salt-induced "physiological dryness" may not always be soundly based. Since, in nature, the osmotic pressure of the soil water is unlikely to change at rates much in excess of the 1 atm per day assumed in the numerical example above, it appears that the direct osmotic effect on plant water uptake is unlikely to be large, provided that liquid phase continuity between the soil water and the root surfaces is maintained. (We discuss this proviso below.) The basis of the deleterious effect of soil salinity in fairly moist soils may need to be sought elsewhere. It is possible that in such cases symptoms which have been interpreted as due to "physiological drought" have in reality been caused by the entry of diffusible solutes in toxic quantities.

Study of the movement of soil water to the absorbing root system (18) indicates that, during transpiration, large moisture gradients may develop near the root surfaces. As a result, even at fairly high mean soil moisture contents, the soil immediately adjoining the absorbing surface may become so dry that the final transfer of water to the root must take place in the vapor phase (17). The failure of continuity may be aggravated by the shrinkage of the root cells as they lose turgor in a drying soil. The vapor gap produced operates as an ideal semi-permeable membrane. Thus, a moderately dry soil, combined with meteorological conditions which impose a high transpiration rate upon the plant, may produce a situation in which the concept of "physiological drought" becomes relevant.

Walter (26) states, "Formerly it was assumed that salty soils, through the osmotic effects of the salts, are physiologically dry for plants. But this osmotic effect

is balanced by the intake of salts." Walter gives no qualification or further explanation of this statement. This total rejection of the physiological drought concept, like its total acceptance, appears to be incorrect. His statement might, however, be accepted by persons working with water cultures. In these no continuity problem arises, and experiment tends to confirm the analysis developed here (13).

The possibility of solute diffusibility makes the term "effective soil moisture stress" useful. We define it here as the minimum value of $(P_n - T)$ in the root cells necessary to produce movement from the soil into the plant. It will be recalled that P_n is the osmotic pressure of the cell contents due to non-diffusible solutes. Now, since diffusible solutes may be present in the root cells as well as in the soil water, we may write, quite generally,

$$\Psi' = \Psi + P_i - P_d \qquad (22)$$

where Ψ' is the effective soil moisture stress (atm), and Ψ is the soil moisture tension (atm). P_i, P_d now represent the osmotic pressure of the soil solution and the partial osmotic pressure of the cell contents due to diffusible solute, respectively. Let θ be the volumetric moisture content of the soil (cm^3 liquid water/cm^3 soil) and θ_f the value of θ at which liquid continuity fails. Then

$$\text{for } \theta > \theta_f,\ P_i \approx P_d, \text{ so that } \Psi' \approx \Psi \qquad (23)$$

When $\theta \leqslant \theta_f$ matters are more complicated, as the quantity of diffusible material in the plant may depend on the salinity history of the root zone before failure of continuity. When, for example, all the salt accumulates in the soil after the continuity failure (we shall call this the limiting case A),

$$P_i = P_s\theta_s/\theta,\ P_d = 0 \qquad (24)$$

where P_s denotes the osmotic pressure (atm) of the soil solution, if the soil were at the saturation moisture content θ_s and contained the same amount of salt per unit soil volume. Equation 24 assumes proportionality between osmotic pressure and solute concentration. This gives the result

$$\text{Case A} \quad \text{for } \theta \leqslant \theta_f,\ \Psi' = \Psi + P_s\theta_s/\theta \qquad (25)$$

In this case Ψ' would agree with Wadleigh's "total soil moisture stress" in the range $\theta \leqslant \theta_f$.

If, on the other hand, we consider the contrasting case B, where the salt in the soil is present in constant (volumetric) concentration before, as well as after the failure of continuity, equation 24 must be replaced by

$$P_i = P_s\theta_s/\theta,\ P_d = P_s\theta_s/\theta_f \qquad (26)$$

(The expression for P_d neglects the effect of turgor changes in the root and may therefore be somewhat too small.) Equations 22 and 26 then yield

$$\text{Case B} \quad \text{for } \theta \leqslant \theta_f,$$

$$\Psi' = \Psi + P_s\theta_s\left(\frac{1}{\theta} - \frac{1}{\theta_f}\right) \qquad (27)$$

This case gives a Ψ' which gradually deviates from Walter's concept of $\Psi' = \Psi$ as θ decreases below θ_f, but which everywhere remains somewhat less than Wadleigh's "total soil moisture stress."

In general terms we may state that, according to the present ideas, $\Psi' = \Psi$ so long as $\theta > \theta_f$. For $\theta \leqslant \theta_f$ the exact behavior of Ψ' depends on the salinity history of the root zone, but, in general, Ψ' can be expected to assume a value intermediate between that implied either by total acceptance or total rejection of the physiological drought concept.

Figure 6 illustrates the $\Psi'(\theta)$ relationships implicit in the physiological drought theory and in the opposing view of Walter, as well as the relationships for Cases A and B according to the present considerations. The $\Psi(\theta)$ function was based on data of Moore (15) for a soil of moderate clay content, and the value $P_s = 5$ was used.

Summary

The dynamic theory of the classical osmotic plant cell is developed in quantitative form and extended to the case where a diffusible solute is present. It is shown that solute diffusibility may result in marked deviations from classical behavior. Where Donnan membrane phenomena are operative, the analysis needs modification, but the gross character of the dynamic behavior remains similar. The "physiological drought" theory of the influence of soluble salts in the soil on the water economy of plants depends on their non-diffusibility. On the other hand, Walter (26) states, "the osmotic effect is balanced by the intake of salts." Neither view seems wholly correct, since both solute diffusibility and liquid phase continuity may be important factors.

The author acknowledges the helpfulness of criticisms by his colleagues, both in C. S. I. R. O. and C. I. T.; in particular those of Mr. C. I. Davern and Dr. James Bonner.

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PROPAGATION OF TURGOR AND OTHER PROPERTIES THROUGH CELL AGGREGATIONS [1]

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Inferences are often drawn about the osmotic behavior of pieces of tissue composed of an aggregation of cells, or even of whole plants, by reference to the classical single cell osmometer. Treating the aggregate as an individual introduces no error when the system is in internal equilibrium. However, in the dynamic problems of interest to the physiologist, this is not the case.

We consider first the behavior of a linear aggregation of cells, as shown in figure 1. The cells are identical in the sense that, at zero turgor, their dimensions, and the osmotic pressure of their contents, are equal, and that the elastic and permeability properties of their walls and membranes are equal. A is the effective area of wall available for the passage of water between adjoining cells (cm^2), K is the permeability of the surface of an individual cell to water ($cm\ sec^{-1}\ atm^{-1}$), θ is the diffusion pressure deficit (atm), T is the cell turgor pressure (atm), P is the osmotic pressure of the cell contents (atm), V is the cell volume (cm^3), t is the time (sec), and numerical suffixes denote values obtaining in the appropriately numbered cell. Then, for any sequence of cells, 1, 2, 3,

$$\left.\begin{aligned}\text{Rate of flow of water from 1 to 2} &= \frac{AK}{2}(\theta_2 - \theta_1)\\ \text{Rate of flow of water from 2 to 3} &= \frac{AK}{2}(\theta_3 - \theta_2)\end{aligned}\right\} \quad (1)$$

In attributing definite values θ_1, θ_2, θ_3 to the diffusion pressure deficits of cells 1, 2, 3, we imply that osmotic pressure differences within each cell are negligible. Commonly the cell dimensions, and the rates of water transfer, will be so small that this is the case.

It follows that

$$\text{Rate of volume increase of cell 2} = \frac{AK}{2}[(\theta_2 - \theta_1) - (\theta_3 - \theta_2)] \quad (2)$$

By introducing a relationship between V and θ, we could now write down a differential equation express-

[1] Received February 27, 1958.
[2] Address for 1958. This work was supported in part by a grant from the Rockefeller Foundation, RF 56160.
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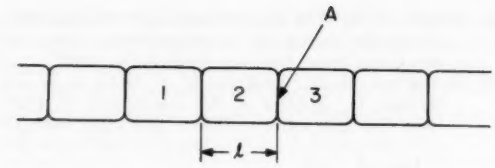

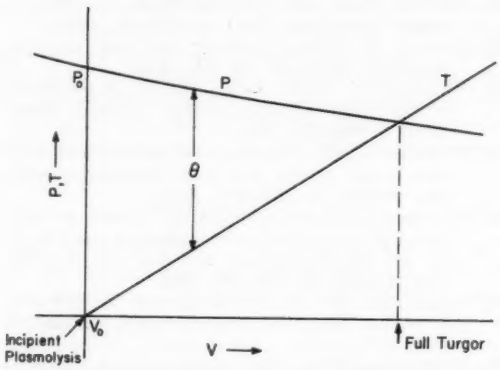

FIG. 1 (*top*). Linear aggregation of osmotic cells.

FIG. 2 (*bottom*). Simplified Höfler diagram. Cell osmotic relations connecting osmotic pressure, P, turgor pressure, T, diffusion pressure deficit, θ, and cell volume, V.

ing $d\theta_2/dt$ in terms of θ_1, θ_2, and θ_3. We could do similarly for each cell of the aggregation. However, since we should then have 1,000 simultaneous differential equations for an aggregation of 1,000 cells, and 1,000,000 equations for 1,000,000 cells, it is simpler and more elegant to use the fact that individual cells are much smaller than the aggregation, and to treat θ as varying continuously along a distance ordinate x directed lengthwise along the aggregation. Then if l is the length of each cell (cm), measured in the x-direction, $[(\theta_2-\theta_1)-(\theta_3-\theta_2)]/l^2$ represents in finite difference form (e.g., (3)) the quantity $-\partial^2\theta/\partial x^2$. Thus eq. (2) reduces to

$$\frac{\partial V}{\partial t} = -\frac{AKl^2}{2}\frac{\partial^2\theta}{\partial x^2} \qquad (3)$$

Figure 2 shows in simplified form the well-known relationships between V, P, T and θ. When V is nearly a linear function of θ, it is readily shown (7) by using the fact that cell volume changes are relatively small, that, to a good approximation,

$$\theta = (\epsilon + 2P_o) - \frac{V}{V_o}(\epsilon + P_o) \qquad (4)$$

where V_o, P_o are the values of V, P for $T = 0$ (incipient plasmolysis) and ϵ is the elastic molulus defined by the equation

$$T = \epsilon(V/V_o - 1) \qquad (5)$$

Note that equation 4 implies that the cell volume at full turgor ($\theta = 0$) is

$$V \qquad V_o\left[1 + \frac{P_o}{\epsilon + P_o}\right]$$

As we state in (7), the assumption of linear relations between T and V, and θ and V, is a first approximation. We show later that a more sophisticated analysis permitting any arbitrary form of T(V) and θ(V) functions may be readily developed. It will also be clear from (7) that the present study deals only with elastic changes of cell volume, and does not treat the plastic, irreversible deformations associated with growth.

Combining equations 3 and 4,

$$\frac{\partial V}{\partial t} = \frac{AKl^2(\epsilon + P_o)}{2V_o}\frac{\partial^2 V}{\partial x^2} \qquad (6)$$

The equation is clearly of the diffusion form

$$\frac{\partial V}{\partial t} = D\frac{\partial^2 V}{\partial x^2} \qquad (7)$$

The diffusivity D is a function of K, $(\epsilon + P_o)$ and the cell dimensions. Since Al/V_o is a function of cell shape, and is independent of cell size, it may be replaced by a shape factor a. It follows that

$$D = \frac{aKl(\epsilon + P_o)}{2} \qquad (8)$$

For plant cells $(\epsilon + P_o)$ is typically of order of magnitude 100 atm (7). a is about 1; it is somewhat less if (gas-filled) free spaces exist between the cells.

Since V, T, θ and P are all assumed to be connected linearly, equation 7 can be recast as a diffusion equation in the other quantities. We may therefore write also

$$\frac{\partial T}{\partial t} = D\frac{\partial^2 T}{\partial x^2}; \frac{\partial \theta}{\partial t} = D\frac{\partial^2 \theta}{\partial x^2}; \frac{\partial P}{\partial t} = D\frac{\partial^2 P}{\partial x^2} \qquad (9)$$

and D is equally the coefficient of diffusion of turgor, diffusion pressure deficit, or osmotic pressure, as well as being the coefficient of diffusion of cell volume. (θ(t) will often be fixed at the boundaries, so that the θ-form of the equation will probably be the most serviceable.)

The analysis is simply generalized to three-dimensional aggregations by applying the same considerations in two further directions at right angles to the x-direction and to each other. Where the tissue is isotropic (i.e., a and l are independent of direction in the tissue) the diffusion equations assume the general form

$$\frac{\partial V}{\partial t} = D\nabla^2 V, \text{ etc.} \qquad (10)$$

Where the cells are of different dimensions in the mutually perpendicular principal directions x, y, z, the equations are of the form

$$\frac{\partial V}{\partial t} = D_x \frac{\partial^2 V}{\partial x^2} + D_y \frac{\partial^2 V}{\partial x^2} + D_z \frac{\partial^2 V}{\partial z^2}, \text{ etc.} \quad (11)$$

Here D_x, D_y and D_z are not necessarily equal, each being specified by an equation similar to 8, but with values of a and l appropriate to the particular principal direction in the tissue. (In this more general case the diffusivity becomes a second-order tensor of a form common in the mathematical physics of anisotropic media.)

When the arrangement of cells is other than rectangular, these three-dimensional results do not follow directly from the one-dimensional results. However, provided the tissue is homogeneous (i.e., the individual cells are similar and their spacing is uniform) results of the same nature can be anticipated, though the relationship between the D's and the cell dimensions may now become more complicated.

Some Limitations and Possible Extensions: It remains to mention some further aspects of the present approach, mainly dealing with its limitations and its possible extensions.

1. Our analysis deals solely with water exchange between adjoining cells. It must be recognized that, at least in the three-dimensional case of greatest practical interest, there exists the possibility of passage of water between non-adjoining cells through water-occupied intercellular spaces. Fortunately for the present approach, intercellular spaces in plants are normally gas-filled, so that this difficulty will not usually arise.

2. We have not attempted a detailed analysis of the stress-distribution in the framework of cell-walls within the tissue. Obviously the exact stress picture in the structure wall—middle lamella—wall between two cells of differing turgor pressure will depend on the elastic properties of the various components in a manner which may be far from simple. Two extreme working hypotheses are (a) that the middle lamella does not support stress, in which case the cells are elastically independent, and (b) that the two walls plus the middle lamella possess a common tension which depends on the mean of the turgor pressure in the two cells. The present treatment agrees exactly with hypothesis (a) and also, to a first order of small quantities, with hypothesis (b). To this degree elastic interactions between the cells are taken into account. Obviously this aspect needs further study.

3. Here we examine further the approximation that D is constant in a particular tissue. This result depends on (a) neglect of the small changes in l which occur with changes in turgor, and (b) the assumption of a linear relationship between V and the quantity regarded as diffusing (i.e., θ, T or P).

(b) is the more drastic assumption, since it is well known that the Höfler diagram may reveal quite appreciable deviations from linearity. We show here, however, that a more exact expression for D can be found simply in terms of any given Höfler diagram.

In the more precise analysis equation 3 is replaced by

$$\frac{\partial V}{\partial t} = -\frac{\partial}{\partial x}\left(\frac{AKl^2}{2}\frac{\partial \theta}{\partial x}\right) \quad (12)$$

and, instead of using equation 4, we introduce the quantity $d\theta/dV$. Previously with V and θ assumed connected linearly, this quantity was equal to $-(\epsilon + P_o)/V_o$. When the Höfler diagram is non-linear, $d\theta/dV$ is a function of V. Now equation 12 may be rewritten

$$\frac{\partial V}{\partial t} = \frac{\partial}{\partial x}\left(-\frac{AKl^2}{2}\frac{d\theta}{dV}\cdot\frac{\partial V}{\partial x}\right) \quad (13)$$

i.e.,

$$\frac{\partial V}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial V}{\partial x}\right) \quad (14)$$

where

$$D = -\frac{AKl^2}{2}\frac{d\theta}{dV} \quad (15)$$

Equations 13, 14 and 15 correspond to equations 6, 7 and 8 of the linear analysis. As before, we may develop the extensions to three dimensions for both isotropic and anisotropic tissue. It is no longer possible, however, to express the phenomenon as diffusion of θ, T and P, as well as of V. The relevant equations assume the slightly more complicated "heat conduction" form. D is now, evidently, a function of V, and if the manner in which Al^2 depends on V is known, there is no formal difficulty in avoiding approximation (a) as well as approximation (b).

It will be noted that equations 14 and 15 define a diffusion phenomenon with the diffusivity concentration-dependent. The mathematics of this form of diffusion has been considerably advanced in recent years (cf. 2, 6), one of the practical applications being to the problem of water movement in unsaturated soils.

However, it is our view that the linear theory is sufficiently refined for most plant physiological purposes, at least until uncertainties such as those mentioned in 1 and 2 above are resolved. For example very large deviations from linearity in the Höfler diagram would be needed before D would be found to vary in a range of V from, say, 50 to 200 % of some mean value. Yet, even in this extreme case, errors due to using D constant would probably be less than other uncertainties in the problem. Contrast the case of water movement in soils, where D may vary by 1 to 1000, or more, and it becomes much more necessary to take into account the variation of D with concentration.

4. The deformation of the tissue, produced by any given osmotic treatment, can be inferred, once the problem is solved for V, provided always that the relationship between V and the geometry of the individual cell is known. (If the cell deforms isotropically, its external dimensions will vary as $V^{1/3}$.)

5. The analysis, in its present form, does not apply to the case where diffusible salts are present in the

tissue, since P_o is then constant, neither in time nor space.

6. Further, it is valid only for turgid cells, since most of the relations between V, T, θ and P, which we use, hold only for the normal unplasmolyzed cell.

OSMOTIC DIFFUSION AND SELF-DIFFUSION: Molecular and ionic diffusion in tissue is usually complicated by osmotic effects. However, these are apparently absent where the diffusing substance is isotopically labelled water (1, 4, 5). In such cases we may use the methods of the above analysis to arrive at a value for the apparent diffusivity of the labelled water (and, presumably, the apparent coefficient of self-diffusion of H_2O) through the tissue, $D_s(cm^2sec^{-1})$, namely

$$D_s = \frac{\alpha K_s l}{2}, \qquad (16)$$

where K_s is the permeability of the surface of an individual cell (cm sec^{-1}) to diffusion of the labelled water.

I am indebted to Dr. James Bonner for raising the question of the relationship, if any, between the apparent coefficients of osmotic diffusion, D, and of self-diffusion, D_s. The experiments of Ordin and Bonner (5) suggests $D/D_s \approx 1$, while the somewhat less precise (5) work of Buffel (1) gives $D/D_s \approx 0.1$.

According to the present analysis (equations 8 and 16):

$$\frac{D}{D_s} = \frac{K(\epsilon + P_o)}{K_s} \qquad (17)$$

D involves $(\epsilon + P_o)$, i.e., a quantity depending on both the amount of osmotically active material in the cell and the elastic properties of the cell, while D_s does not. (The argument is, of course, the same when we use equation 15 for D; in qualitative form, the argument is simply that D depends on the Höfler diagram, while D_s does not.) It would seem to be purely coincidence that the two diffusivities are of about the same order of magnitude.

No simple quantitative relationships connecting either D and D_s, or K and K_s, seem possible. Studies of diffusion of labelled water have an interest in their own right, but cannot be regarded as a substitute for osmotic studies. Permeability to water in the osmotic sense, and in the self-diffusion sense, should not be confounded.

SUMMARY

The problem of the propagation of osmotic disturbances through tissue consisting of an aggregation of osmotic cells is analyzed. It is shown that such disturbances can be described mathematically as diffusion phenomena, the diffusivity being a function of the dimensions, permeability, and elastic properties of the individual cell, together with the quantity of osmotically active material in it. The problem of diffusion of isotopically labelled water through such tissue may be treated similarly. The diffusion coefficients of the two phenomena depend on different properties of the system. The experimental result that they are of about the same order of magnitude is, apparently, merely a coincidence, and does not substantiate the idea that diffusion studies of labelled water can give information directly connected with cell water relations.

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OSMOSIS AND DIFFUSION IN TISSUE: HALF-TIMES AND INTERNAL GRADIENTS[1]

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THE INDIVIDUAL CELL: The use of the "half-time" to describe the dynamics of diffusion between an individual cell and a large body of solution in which it is placed seems to have originated with Collander and Bärlund (4). The concept depends on the primary resistance to diffusion residing in the membrane or wall enclosing the cell. It is simply shown that, if such a cell is suddenly immersed in the solution, the concentration of solute inside the cell, θ, is given by the equation

$$\theta = \theta_0 + (\theta_1 - \theta_0)\,[1 - \exp(-K_s At/V)] \qquad (1)$$

where θ_0 is the initial concentration of solute within the cell, θ_1 is the concentration in the external solution, K_s is the permeability of the cell surface to the solute (cm sec^{-1}), A is the area of the cell (cm^2), V is the volume of the cell (cm^3) and t is the time (sec), with the origin of time at the instant of immersion of the cell in the solution. It follows that approach to the new equilibrium concentration within the cell is half accomplished at

$$t = t_{1/2} = (\log_e 2)V/K_sA = 0.693\ V/K_sA \qquad (2)$$

and $t_{1/2}$ is called the "half-time." The analysis neglects changes in A and V due to osmotic effects, so that it might be expected to describe self-diffusion (e.g., diffusion of labelled water) more closely than the diffusion of other solutes.

A quite analogous result concerning "osmotic diffusion" (8) has been described in reference (7). Essentially, we may state that if the θ's in equation 1 now denote diffusion pressure deficits instead of concentrations, and K_s is replaced by $K(\epsilon + P_0)$; equation 1 represents to a good approximation the dynamics of diffusion pressure deficit change of a cell suddenly immersed in a large body of solution at a different diffusion pressure deficit. In the preceding sentence, K is the permeability of the cell surface to water (cm sec^{-1} atm^{-1}), ϵ is the elastic modulus of the cell (atm) (7, 8), and P_0 is the osmotic pressure of the cell contents at zero turgor (atm). θ_0 is, in this revised symbolism, the initial diffusion pressure deficit of the cell contents, and θ_1 is that of the external solution.

TISSUE: Diffusion and osmotic phenomena in whole pieces of tissue have often been assumed to follow a course quite analogous to that in a single cell. This is not justified, since the resistance to propagation of the disturbance is not confined to the tissue boundaries, but has its seat in the cell membranes distributed throughout the tissue. As a result, very marked differences in concentration (or diffusion pressure deficit, or turgor) will exist within the tissue during the course of equilibration, whereas the individual cell model assumes that the concentration (etc.) within the boundaries is everywhere the same. One aim of the present study is to show just how large these gradients within the tissue can be expected to be. It should be noted also that, when applied to tissue, the half-time no longer carries with it the implication that the process is exponential. (We return to this matter in the section below entitled "Cumulative uptake curves.")

Nevertheless, the half-time remains a graphic and convenient means of summarizing the dynamics of these processes in this more complicated case where the cell is replaced by a piece of tissue. It is important, however, that the effect of geometry on half-times be understood, and that, if possible, a parameter describing the dynamics of the process be established which is independent of tissue geometry, and is therefore of a more fundamental nature. Such a parameter does, in fact, exist—the diffusivity, or diffusion coefficient, D.

The correspondence between osmotic diffusion and self-diffusion, noted for the single cell, carries over to the case of a piece of tissue consisting of an aggregation of cells. It is shown elsewhere (8), by treating the individual cells as small compared with the tissue as a whole and regarding the diffusing property as varying continuously, that both phenomena can be described to a first approximation by a diffusion equation, the ratio between the two appropriate diffusivities being $K(\epsilon + P_0)/K_s$.

Here we shall not be concerned with the value of the diffusivity, D, nor whether the phenomenon considered is osmosis or self-diffusion. We shall treat D as constant, and regard the tissue as isotropic. Our aim is to analyze the way in which the half-times of tissues as a whole vary with tissue dimensions, and in which the half-times of individual cells vary with position within the tissue, and how these various half-times connect with D. Such a study has obvious relevance to recent physiological work in which observed half-times have referred to tissues of different dimensions, and to cells in various locations (1, 5, 6).

The general problem reduces to finding the solution of the equation

$$\frac{\partial \theta}{\partial t} = D\nabla^2\theta \qquad (3)$$

subject to the conditions:

$$\theta = \theta_0 \text{ in R at } t = 0$$
$$\theta = \theta_1 \text{ at S for } t \geqslant 0 \qquad (4)$$

[1] Received March 3, 1958.
[2] Address for 1958. This work was supported in part by a grant from the Rockefeller Foundation, RF 56160.
[3] Permanent address.

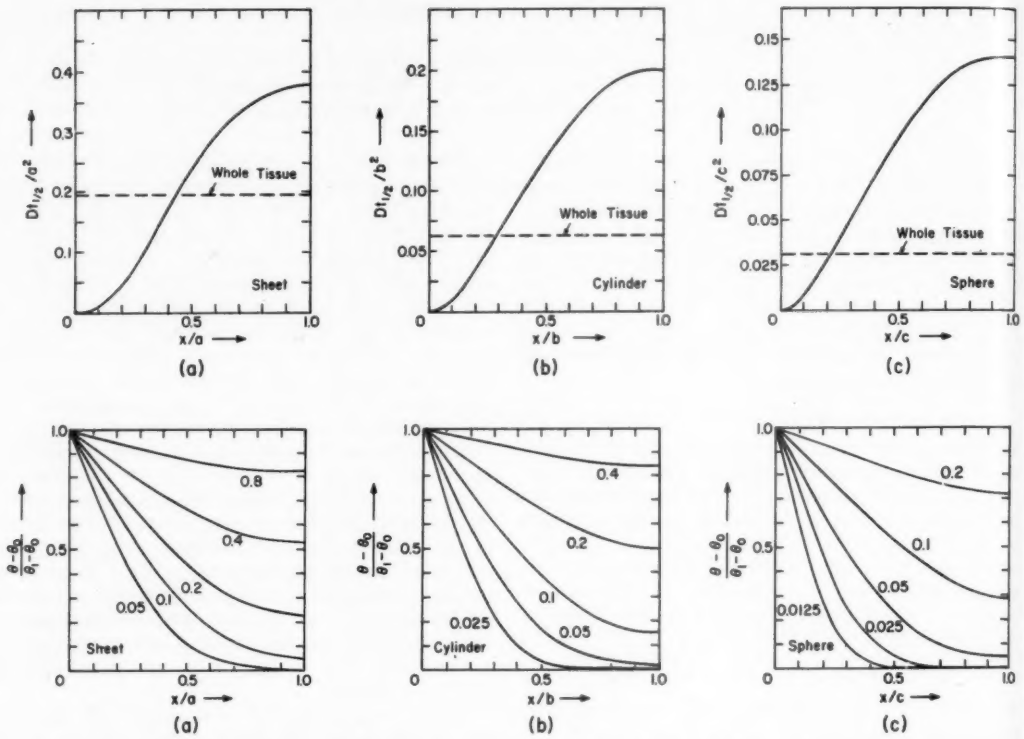

FIG. 1 (*top*). Half-times for equilibration of tissues and individual cells. (a) For plane sheet of tissue, thickness 2a(cm). (b) For cylinder of tissue, radius b(cm). (c) For sphere of tissue, radius c(cm). In each case the broken horizontal curve shows the half-time for the tissue as a whole, and the full curve shows the variation of half-time for an individual cell located x(cm) from the tissue surface. In each case D denotes the diffusivity (cm^2sec^{-1}), and $t_{1/2}$ the half-time (sec).

FIG. 2 (*bottom*). Internal gradients during equilibration of tissue. (a) For plane sheet, thickness 2a(cm). For cylinder, radius b(cm). (c) For sphere, radius c(cm). Numbers on the curves of figures (a), (b) and (c) denote values of Dt/a^2, Dt/b^2, Dt/c^2, respectively. D denotes the diffusivity (cm^2sec^{-1}) and t time (sec). θ denotes concentration (or diffusion pressure deficit—see text), θ_0 being the initial value of θ within the tissue, and θ_1 the value of θ in the external solution.

Here θ may refer to diffusion pressure deficit (osmotic diffusion) or concentration of labelled water (self-diffusion). R denotes the space occupied by the tissue and S its surface. We consider three cases, the sheet, the cylinder, and the sphere, which will illustrate the effects of tissue geometry, and of cell location within the tissue, in a wide variety of circumstances. The treatment depends on well-known results in the mathematics of diffusion. Many further results of interest to physiologists will be found in the standard texts (2, 3).

THE SHEET, THE CYLINDER, AND THE SPHERE: Our first example is of a plane sheet of tissue of thickness 2a(cm). We suppose that the other dimensions of the sheet are large compared with a (or else that the edges of the tissue are impermeable). We may therefore neglect diffusion across the edges, and consider only exchange across the upper and lower surfaces of the sheet. The problem then becomes, essentially, one-dimensional, and we may characterize any cell by x(cm), the distance of the cell center from the nearer of the upper and lower surfaces of the sheet. The variation of apparent half-time of cell behavior with cell location, as well as the half-time of the tissue as a whole, is shown in figure 1 a. In figure 2 a are shown profiles of θ within the tissue at various times during the process of equilibration with the external solution.

We consider next tissue in the form of a cylinder of radius b(cm), with its length large compared with b (or else with the cylinder ends impermeable). We neglect diffusion across the ends, and consider only exchange across the cylindrical surface. The problem is then a two-dimensional one with radial symmetry, and we may characterize any cell by x(cm), the radial distance of the cell center from the cylindrical

surface. Figures 1 b and 2 b give the results for this case which correspond to those for the sheet shown in figures 1 a and 2 a.

Finally, we give in figures 1 c and 2 c the corresponding results for a sphere of tissue of radius c(cm). In this case x(cm) is the radial distance of the cell centers from the surface of the sphere.

It will be noted that in any actual tissue, half-times and values of θ will vary discontinuously from one cell to the next. Thus each smooth curve of figures 1 and 2 will be replaced by an approximating histogram or step-function, a step occurring at each boundary between two cells.

We may draw the following conclusions from figures 1 and 2: 1) The half-time for equilibration of an individual cell is very sensitive to cell position, and may vary in value from a very small fraction of the half-time of the tissue as a whole to over four times the value for the whole tissue. 2) The half-time of two tissues of identical shape, but differing in size, will vary as the square of the ratio of their dimensions: e.g., the half-time of a sheet, cylinder or sphere with thickness or radius twice that of another sheet, cylinder or sphere will be four times that of the smaller one. 3) The effect of tissue shape on half-time may be illustrated by the result that the half-times of a sheet, a cylinder and a sphere, each with unit semi-thickness or radius, are in the ratio 0.195 : 0.062 : 0.031 ($2\pi : 2 : 1$?). 4) Large internal gradients may exist within the tissue. Thus, in the sphere, a substantial region of tissue around the center of the sphere is still 25 % removed from equilibrium after a time equal to 6 (tissue) half-times has elapsed.

Cumulative Uptake Curves: By "cumulative uptake" we mean the cumulated total flux into (or out of) the cell or tissue. Often this is best expressed for comparative purposes in terms of $\bar{\theta}$, the instantaneous mean value of θ within the cell or tissue. We may regard the variable $(\bar{\theta}-\theta_0)/(\theta_1-\theta_0)$ as a dimensionless measure of progress of the process of equilibration. This quantity is 0 at t = 0 and 1 at equilibrium.

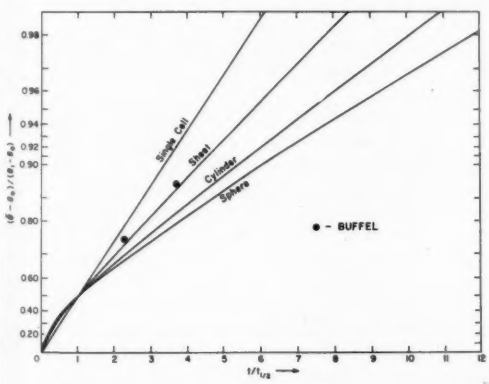

Fig. 3. Cumulative uptake curves for the single cell, the plane sheet, the cylinder, and the sphere. t is the time (sec) and $t_{1/2}$ the half-time (sec). θ denotes concentration (or diffusion pressure deficit—see text), θ_0 being the initial value of θ within the tissue, θ_0 the value of θ in the external solution, and $\bar{\theta}$ the mean value of θ within the tissue. Buffel's experimental data are also shown.

The cumulative uptake curves for the single cell and for tissues of various shapes all differ. Only for the single cell is uptake exactly exponential. In all other cases the rate of approach to equilibrium decreases more rapidly with time than does an exponential process. In figure 3 we compare cumulative uptake curves for the single cell, and for cell aggregates of the three shapes—sheet, cylinder, and sphere. Time scales are made comparable by using the half-time in each case as the unit.

Self-diffusion in Avena Coleoptile Sections: Self-diffusion into Avena coleoptile sections has been observed by Buffel (1) to exhibit this type of deviation from exponential behavior. The data of Ordin and Bonner (6), both for self-diffusion and for osmotic diffusion into Avena coleoptile slices, reveal similar deviations from exponential behavior. Buffel's mean points for 35 sections are plotted on figure 3, and indicate that his sections behaved as "sheets," in our present terminology. The data of Ordin and Bonner follow the same curve within the limits of experimental error.

Superficially this may seem surprising, as one might have expected these cylinders of finite length to behave in a manner intermediate between "cylinders" and "spheres." The probable explanation, which we accept here, is that the cylindrical cuticular surface of the coleoptile is impermeable in comparison to the cut surfaces and to the internal walls and membranes of the tissue. Thus, entry via the cut surfaces only is important, and the section behaves as a sheet.

Buffel used 4-mm sections and obtained half-times for self-diffusion of about 5.5 minutes. Ordin and Bonner used 5-mm sections and obtained half-times for self-diffusion of about 9 minutes. The results of figure 1 a enable us to recast both observations in terms of the apparent coefficient of self-diffusion through Avena coleoptile tissue. We have

$$D = \frac{0.195\, a^2}{t_{1/2}} \qquad (5)$$

Buffel's $a = 0.02$, $t_{1/2} = 330$ yields $D = 2.36 \times 10^{-7}$ $cm^2 sec^{-1}$. Ordin and Bonner's $a = 0.025$, $t_{1/2} = 540$ yields $D = 2.26 \times 10^{-7}$ $cm^2 sec^{-1}$. Thus, when the results are reduced to a common basis by use of the fundamental parameter, D, it is seen that the two investigations are in remarkably good agreement, both indicating $D = 2.3 \times 10^{-7}$ $cm^2 sec^{-1}$—i.e., about 1 % of the coefficient of self-diffusion in free water. (Note that the data of Ketellapper (5) cannot be analyzed in this way, since he mounted 12 sections on the spindle of an electromagnetic diver. The rate of exchange between his sections and the external solution clearly depends on the tightness of packing of the

sections (or, rather, on the dimensions of the gaps between the cut surfaces of neighboring sections).)

Summary

The variation with tissue dimensions of the half-times of osmosis and self-diffusion, the manner in which half-times of individual cells vary with cell position within the tissue, and the internal gradients within tissue during such phenomena are analyzed. These processes may be described mathematically by means of a diffusion equation, and the basic parameter characterizing the dynamics of the process is the diffusivity, D. The data of Buffel (1) and of Ordin and Bonner (6) on self-diffusion in Avena coleoptile sections indicate that the cylindrical cuticular surface of the section is relatively impermeable and that exchange is principally across the cut surfaces. Ordin and Bonner's data suggest a similar result in the case of osmotic water exchange as well. The data of Buffel and those of Ordin and Bonner on self-diffusion are for Avena coleoptile sections of different dimensions, and yield different half-times. When both results are reduced to a value of D, the two agree very closely, yielding $D = 2.3 \times 10^{-7}$ $cm^2 sec^{-1}$, i.e., roughly 1 % of the value for self-diffusion in free water.

I am indebted to Dr. James Bonner for discussion of this topic.

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NON-METABOLIC UPTAKE OF IONS BY BARLEY ROOTS[1,2]

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In dealing with the mechanism of absorption, a major concern is the amount and behavior of the non-metabolic uptake. Not only may it exceed the magnitude of the metabolic absorption but also the absorption process may occur primarily from this phase. Non-metabolic uptake of cations includes an adsorption component in addition to that present in the regions freely accessible to both cations and anions. For anions, non-metabolic uptake may approximate the free space of the root. The concept of free and apparent free space in roots (2, 10) has been given considerable attention, especially in its relation to absorption and translocation of solutes (3, 6, 9, 11, 18, 20). However even with anions, the values of apparent free space obtained for a given root material are dependent upon the methods used for their determination (4).

In the experiments to be reported here, the magnitude of the non-metabolic uptake of two cations and an anion were determined by the difference between the measured metabolic component and the total uptake.

It has been shown that the metabolic absorption of anions is decreased in solutions of high pH (15). This is especially marked for $HPO_4^=$ (7, 8). By using an appropriate pH, the metabolic absorption of this ion is essentially nil and all of its uptake is non-metabolic. Metabolic absorption of cations is little affected by high pH (15). Under these conditions, excess cation absorption occurs. In young, actively metabolizing barley roots, excess cation absorption is balanced by the production of organic acid anions and by replacement of previously absorbed cations. In the absence of anion absorption, the sum of the organic acid increase and the loss of previously absorbed cations is equivalent to the amount of test cation absorbed (13, 14). In these studies, the roots of 6-day-old barley plants were used with K_2HPO_4 and $NaHCO_3$ as test salts. Bicarbonate does not serve to balance cations within this type of root material (14). The non-metabolic uptakes of K^+, Na^+ and $HPO_4^=$ were examined.

[1] Received March 6, 1958.

[2] This paper is based on work performed under contract No. AT-(11-1)-34, Project 5, with the Atomic Energy Commission.

Materials and Methods

Most of the experiments were conducted with excised barley roots, grown and prepared as previously described (16). The variety of barley used was California Mariout, 1956 crop. Although the seedlings were grown in a solution many times more dilute than the test solutions, the excised root material was thoroughly washed in distilled water prior to treatment. All experiments were conducted at 25° C with suitable aeration. Analyses were performed on lyophyllized material. Sodium and potassium were determined by means of the flame photometer. Phosphorus was determined by wet ashing the sample with sulfuric acid and 30 % H_2O_2 and utilizing the molybdenum blue colorimetric procedure. The ethylenediamine tetraacetic acid titration procedure of Cheng and Bray (5) was used for calcium plus magnesium. Organic acids were determined by ether extraction and titration (14).

Results

K^+ Uptake: In the first experiment, samples of washed roots were placed in solutions containing 20.5 millimoles K_2HPO_4 per liter, pH 8.7, for various time periods up to one hour. Twenty grams of roots were used in 15 liters of solution. At the conclusion of the treatment period, the roots were separated onto a Nylon mesh, centrifuged briefly without washing and immediately frozen with liquid N_2 to stop any transference of solute from the non-metabolic to the metabolic phase. After lyophyllization, the roots were analyzed for K^+, Ca^{++} plus Mg^{++}, organic acids and total phosphorus. The non-metabolic uptake was calculated by subtracting the sum of the organic acid increase and the Ca^{++} plus Mg^{++} decrease from the total K^+ uptake, on the assumption that K^+ replaced Ca^{++} plus Mg^{++} lost from the metabolically absorbed fraction. Approximately four minutes elapsed between the removal of the roots from the solution and their freezing. To correct for the small amount of transference from the non-metabolic to the metabolic phase, a correction factor based upon the rate of metabolic absorption was applied. Also, weighing experiments involving length of centrifugation indicated a small retention of the solution and the results were correspondingly corrected. Both these corrections were small and were applied to all data whenever appropriate.

The values of non-metabolic uptake of K^+ for the various treatment times are presented in table I. These values are expressed as the ratio of the non-metabolic uptake to external solution concentration. It is evident from table I that the non-metabolic uptake of K^+ from K_2HPO_4 increases with time at least up to one hour. In general, the values obtained by leaching the treated root material with distilled water are smaller than those obtained from metabolic absorption determinations.

$HPO_4^=$ Uptake: In the same experiment, phosphorus contents were determined. The phosphorus contents were essentially constant in all treated samples, indicating that equilibrium had been reached within five minutes and that there was no significant metabolic absorption of $HPO_4^=$ even at the end of an hour. These observations are given in table II in the column headed "excised roots." The values are the total uptake of phosphorus expressed as the ratio of the total uptake to external solution concentration. Other data showed that the value was affected slightly by the concentration of the solution. At low concentrations, e.g., 2.5 millimoles K_2HPO_4 per liter, the value was slightly smaller than that obtained at the higher concentration.

In another experiment plants were grown on cheesecloth stretched over beakers. The plants were grown similarly to those used for the excised root experiments. The roots were washed and drained by

Table II

Non-metabolic Uptake of $HPO_4^=$ by Barley Roots from 20.5 Millimoles/L K_2HPO_4, Expressed as the Ratio of the Non-metabolic Uptake to External Solution Concentration

Duration of treatment. Minutes	Non-metabolic uptake ratio	
	Excised roots	Intact roots
5	0.308	0.156
15	0.313	0.193
30	0.311	0.209
45	0.323	0.203
60	0.317	0.203

Table I

Effect of Duration of Treatment on the Non-metaeolic Uptake of K^+ by Excised Barley Roots from 20.5 Millimoles/L of K_2HPO_4

Duration of treatment. Minutes	Total K^+ uptake meq/kg	Organic acid increase meq/kg	$Ca^{++} + Mg^{++}$ lost meq/kg	Non-metabolic uptake ratio *	Leachable fraction ratio *
5	12.0	0.7	2.1	0.224	0.236
15	16.3	0.6	2.1	0.332	0.281
30	20.2	3.0	2.6	0.356	0.283
45	21.1	3.3	2.6	0.371	0.305
60	22.7	3.4	2.6	0.407	0.335

* Expressed as the ratio of the non-metabolic uptake or leachable fraction to the external solution concentration.

TABLE III

RELATIONSHIP BETWEEN $NaHCO_3$ CONCENTRATION AND NON-METABOLIC UPTAKE OF Na^+ IN ONE HOUR BY EXCISED BARLEY ROOTS

CONCENTRATION MEQ/L	TOTAL Na^+ UPTAKE MEQ/KG	ORGANIC ACID INCREASE MEQ/KG	K^+ LOST MEQ/KG	$Ca^{++} + Mg^{++}$ LOST MEQ/KG	NON-METABOLIC UPTAKE RATIO *	LEACHABLE FRACTION RATIO *
5.25	11.8	5.8	0.7	1.1	0.800	0.895
10.5	17.2	7.0	1.0	1.3	0.752	0.681
21.0	23.4	6.0	2.3	1.7	0.638	0.658
36.8	30.2	5.8	3.7	1.9	0.512	0.514
52.5	38.8	8.5	4.0	2.5	0.453	0.503

* Expressed as the ratio of the non-metabolic uptake or leachable fraction to the external solution concentration.

decantation without disturbing the arrangement. They were then treated with the same concentration of K_2HPO_4 and for the same time periods as were used in determining non-metabolic uptake by excised roots. Care was taken to avoid injury to the roots. The treatment was carried out in dim light at 25° C. Solutions were aerated. At the end of the treatment period the plants together with the cheesecloth, were wrapped in Nylon mesh and centrifuged. After centrifugation the roots were excised and analyzed. The differences in phosphorus contents between the roots of untreated and treated plants are the total uptakes. These values, again expressed as ratios, are given in table II under the column headed "intact plants." The values are considerably smaller than those obtained with excised roots.

Experiments with intact plants using labeled $HPO_4^=$ and subjected to the same treatment conditions as above, showed no movement of the solution phosphorus to the shoots during the treatment period. Additional experiments indicated no change in total phosphorus content of the shoots during the course of the treatment.

Since there was no metabolic absorption of $HPO_4^=$, it was possible to determine its non-metabolic uptake by a different method. Twenty grams of excised roots were placed in 100 ml of a solution having an initial concentration of 22.6 millimoles K_2HPO_4 per liter and gently agitated. At the end of an hour a sample of the solution was taken and analyzed for phosphate. The change in concentration of $HPO_4^=$ in the solution was used to calculate the non-metabolic uptake. The value obtained by this method was 0.342. This is in reasonably good agreement with the value obtained by direct analysis of the excised root material in the previous experiment.

Na^+ UPTAKE: The uptake of Na^+ from $NaHCO_3$ (pH 8.3) in one hour at different concentrations was determined. On these samples, Na^+, K^+, Ca^{++} plus Mg^{++}, and organic acids were determined. Non-metabolic uptakes of Na^+ were calculated assuming that the loss of K^+ and Ca^{++} plus Mg^{++} was from the metabolically absorbed fraction. The results are given in table III. Values obtained by leaching the treated root material with distilled water are also given in table III. The concentration of the external solution has a very marked effect on the magnitude of the non-metabolic uptake of Na^+. The magnitude expressed as the ratio of the non-metabolic uptake to external solution concentration decreasing as the concentration increases. A comparison with table I indicates a higher value for Na^+ than for K^+, at an equivalent concentration and time, for both the metabolic absorption and the leaching methods.

DISCUSSION

Free space has been defined as that part of a cell or tissue into which the solute and solvent from the external solution penetrate readily (2). Because of the difficulties of measuring free space directly, the concept of apparent free space has been developed. The definition most used for apparent free space is "that portion of a plant tissue into which substances in solution apparently move by free diffusion" (10). The difference between free space and apparent free space has been discussed by Briggs and Robertson (2). In order to avoid problems associated with cation exchange, frequently a non-electrolyte or more usually an anion is used to estimate apparent free space. In the case of cations, there appears to be some ambiguity as to whether the adsorbed component is to be included as a part of apparent free space. The term non-metabolic uptake as used here includes this fraction and therefore its magnitude is dependent upon the nature and concentration of the test ion as well as the material used.

The values of the non-metabolic uptake are expressed as the ratio of the non-metabolic uptake to external solution concentration. This method of expression is useful when different concentrations of external solution are involved. It has the further advantage in that it does not necessarily imply a physical volume or space. Of the three ions investigated here, only the $HPO_4^=$ non-metabolic uptake could be approximately proportional to a volume.

The methods used to determine apparent free space may not be always applicable for non-metabolic uptake and indeed may not be entirely valid for the former. Determinations of apparent free space based on a rapid initial uptake assume a constant rate of metabolic absorption from zero time. Briggs and Robertson (2) have pointed out that this assumption

is not warranted if the rate of metabolic absorption is appreciable. In experiments in this laboratory using actively absorbing material such as excised barley roots in moderately dilute solutions of KBr, the time curve for the uptake of K^+ has indicated a much smaller apparent free space than generally reported for similar material. On the other hand, if the ion is not metabolically absorbed, this procedure should be quite valid. This is the case with $HPO_4^=$. Blocking metabolic absorption through the use of inhibitors such as DNP or low temperature may involve alterations in the tissue. Exodiffusion and exchange methods may be misleading because of the possibility of ion binding and of leakage or exchange from the metabolically absorbed fraction.

In the experiments reported here, total uptakes were determined after centrifugation for one to two minutes at 65 G to remove adherent solution. Centrifugation was used in preference to blotting since the latter might result in incomplete removal of the adhering solution or might injure the roots with the consequent loss of absorbed ions. Levitt (20) has suggested that a substantial portion of the values obtained for apparent free space is due to a surface film of the test solution which is not removed by centrifugation or blotting. He calculates values of about 6 to 10 % for the apparent free space in roots of several species, after correcting for the postulated surface film. However, we have consistently obtained values of 18 to 20 % after a 10-second wash with distilled water which almost certainly removes any surface film of solution. Furthermore, when the uptake of $HPO_4^=$ was determined by the change in concentration of the ambient solution, the results agreed with those obtained by analysis of the centrifuged root material.

The higher values of non-metabolic uptake of K^+ and Na^+ in comparison with that of $HPO_4^=$ can be explained on the basis of the adsorbed cation component. Roots are known to contain nonmobile anions which act as adsorption sites for cations. Contact phenomena of roots involving cation exchange have been described by Jenny (17). Arisz (1) regards the surface of the cytoplasm as a Donnan system with a preponderance of negatively charged plasma molecules. Williams and Coleman (21) have made direct measurements of adsorbed cations on barley roots. The presence of adsorbed cations also provides an explanation for the relatively high non-metabolic uptakes observed at low concentrations of Na^+. After a 10-second wash with distilled water, the remaining non-metabolic uptake is about 18 to 20 %, irrespective of the concentration of the test solution. Evidently, a substantial portion of the adsorbed cation is rapidly hydrolized by water. Prolonged leaching removes considerably more of the cation. With K^+ from K_2HPO_4, one hour of leaching results in values of non-metabolic uptake smaller than those obtained from metabolic absorption data. The reverse is true for Na^+ from $NaHCO_3$. Probably some adsorbed K^+ is not removed by leaching and some metabolically absorbed Na^+ is lost by leaching.

Although $HPO_4^=$ comes to equilibrium with the excised roots within a short time, the non-metabolic K^+ uptake was increasing even at the end of an hour. Possibly additional cation adsorption sites were being generated during the course of the experiment.

Since the roots were thoroughly washed for 15 minutes with distilled water, the adsorbed cations in the root prior to treatment consisted of H^+ and perhaps some K^+ and Ca^{++} plus Mg^{++}. The values in tables I and III, calculated from metabolic absorption, are on the basis that the K^+ and Ca^{++} plus Mg^{++} replaced by the test cation were from the metabolically absorbed fraction. If any part of these ions came from the adsorbed fraction, then the calculated non-metabolic uptake values would have been larger. In these experiments it has not been possible to distinguish between the loss of absorbed and adsorbed ions and so the calculated values in table I and III must be regarded as minimum values.

Non-metabolic uptake of $HPO_4^=$ is essentially the same as apparent free space for this ion. In excised roots, an equilibrium value of about 0.315 is reached in a short time and is relatively insensitive to concentration. The difference between the behavior of excised roots and roots of intact plants is of considerable interest. The higher non-metabolic uptake suggests that in excised roots, a region exists which is freely available to the external solution and that this region is not readily accessible in roots of intact plants. The region best meeting these requirements appears to be the stele. Jackson (12) has estimated that the percentage of the cross sectional area of the root occupied by the stele is 16 to 30 % in several different types of barley roots. Since the stelar non-metabolic uptake may be relatively larger than that of the cortex, this region could account for the observed difference between excised and intact plant roots. Butler (4) obtained higher apparent free space values with intact plants when DNP was used than by exodiffusion and time differentiation methods. He suggests that the difference may indicate movement into additional regions. It seems likely that the additional region is the stele which has become accessible to the external solution through the action of DNP.

Recently, Kylin and Hylmö (19) have reported the converse of the relationship observed here between excised and intact roots. They found a much smaller apparent free space in excised roots of wheat than in roots of the intact plants, namely 18.0 % as compared with 27.5 %. These workers used labeled sulfate and possibly exchange phenomena may account for the difference in their results and those reported here.

Hylmö (11), Epstein (6), Kramer (18) and others have proposed that ions may move passively by mass flow from the etxernal solution through the free space of the root and into the conducting elements.

On the other hand, Butler (4), van den Honert et al (9), Arisz (1) and Brouwer (3) believe that much or most of the ions reach the conducting system via a metabolic pathway. The $HPO_4^=$ data presented here favor this latter point of view. If the stelar region in intact plants is not readily accessible to the external solution then a barrier, presumably the endodermis, must exist which isolates this region from the free space of the root cortex. Such a barrier precludes any major movement of ions into the stele by mass flow.

Summary

K^+, Na^+ and $HPO_4^=$ have been used to determine non-metabolic uptake in barley roots by difference between total uptake and metabolic absorption. The uptakes of the two cations, K^+ and Na^+, show a large adsorption component which is included as part of the non-metabolic uptake. The non-metabolic uptakes are expressed as the ratio of non-metabolic uptake to external solution concentration and vary from about 0.20 to 0.80 depending upon ion species, concentration and duration of treatment. They were calculated on the basis that any loss of cations initially present occurred from the absorbed fraction and were replaced by the test cation. The figures given are therefore minimum values.

The non-metabolic uptake of $HPO_4^=$ which is equivalent to apparent free space for this ion was determined by two different methods in excised roots: one involving centrifugation, the other change in concentration of the external solution. Both gave similar results. Considerably lower values were obtained for non-metabolic uptake with $HPO_4^=$ in roots of intact plants than in excised roots; 0.205 as compared with 0.315. It is proposed that a barrier exists between the stelar region and the cortex of the root and that this barrier may prevent movement of ions by mass flow from the external solution to the conducting elements in the roots of intact plants.

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CHEMICAL CONSTITUTION OF THE PRIMARY CELL WALLS OF AVENA COLEOPTILES[1,2]

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In studies of the structure and development of primary walls of plant cells, little detailed attention has been given to the non-cellulosic components of the wall. Indeed, as Bonner (6, p. 130) has indicated, only rarely have thorough analyses of plant walls of any type been made. For the most part, characterization of wall constituents has depended on solubility properties and staining tests, the specificities of which are open to doubt. With the development of newer techniques such as paper chromatography, means are now available for a much more accurate characterization of components in the walls than has hitherto been possible.

Avena coleoptiles are frequently the object of studies relating to primary walls and in view of this, a detailed analysis of their walls has been carried out. As will be seen, the results differ quite strikingly in some respects from those obtained by Thimann and Bonner (20) 25 years ago.

METHODS AND RESULTS

Seedlings of *Avena sativa* var. Lanark were germinated on damp Kleenex tissue in the dark at about 25° C. Coleoptiles were harvested when from 20 to 35 mm long. At this length, the parenchyma cells are elongating and have only primary walls (18). A small amount of secondary wall is present in the vascular bundles but was not considered separately in the study. Primary leaves were removed from fresh coleoptiles which were placed in water until sufficient material had been accumulated. The bulk of the water was then removed by suction and the wet coleoptiles weighed. Roughly 200 g of this material was ground with water in a Potter-Elvehjem homogenizer. The larger particulate matter (mainly wall fragments) was centrifuged, washed three times with cold distilled water and dried. The supernatant plus washings and the dried residues were fractionated according to the schemes shown in figures 1 and 2 respectively.

The initial treatment of the coleoptiles just described was designed to remove the bulk of the cytoplasm from the wall fragments and is similar to the scheme employed by Thimann and Bonner (20). The subsequent fractionation was essentially that commonly used for isolating carbohydrates from plant material (23). Throughout the fractionation, evaporations were carried out under diminished pressure at 40° C or less. Solid fractions, both precipitates and residues, were dried by the same procedure except where otherwise stated; the solid was washed several times with absolute ethanol, then with ether, and dried at a pressure of 0.03 mm Hg over anhydrous calcium chloride for at least 18 hours. Hydrolyses were carried out by heating samples of 10 to 20 mg of the various fractions with 1 ml *N* hydrochloric acid in a sealed tube at 100° C for 8 hours. Chromatograms were run by the descending method (17) using one of the following solvent systems: (A) pyridine : ethyl acetate : water—1 : 2 : 2 (11); (B) ethyl acetate : acetic acid : water—3 : 1 : 3 (11); (C) *n*-butanol : pyridine : water—6 : 4 : 3 (10). Sugars were detected on the chromatograms by the *p*-anisidine hydrochloride spray reagent (10) and were chromatographically identified by running samples of known sugars on the same paper sheet. At least two solvent systems were used to establish this identification. Nitrogen was determined on 15 to 25 mg samples by the micro-Kjeldahl method and protein was taken as 6 times the micro-Kjeldahl value.

The supernatant and washings from the ground coleoptiles were dialyzed in Visking cellophane tubing against fresh distilled water for three 24-hour periods. A precipitate which formed inside the dialysis tube was centrifuged, washed once with water and dried to give Fraction 1 (0.5323 g; N, 9.85 % = 59 % protein). After hydrolysis, chromatography (solvents A and C) revealed the presence of arabinose, xylose, glucose and galactose in the approximate ratios shown in table I.

The supernatant liquor from Fraction 1 was concentrated to 1/20 its volume and ethanol was added until precipitation was complete. The precipitate was centrifuged and dried to give Fraction 2 (0.3166 g; N, 5.26 % = 32 % protein). Hydrolysis and chromatography (solvents A and C) showed the presence of arabinose, xylose, glucose and galactose (table I).

The ethanolic mother liquors from Fraction 2 were evaporated to dryness and the residue was redissolved in ethanol. Addition of ether caused precipitation of Fraction 3 which was washed once with ether and dried (0.2360 g; N, 3.6 % = 22 % protein). Arabinose, xylose and glucose were detected chromatographically (solvents A and C) after hydrolysis. Evaporation of the supernatant liquor from Fraction 3 left no residue indicating that Fractions 1, 2 and 3 together represented all of the non-dialyzable material washed from the coleoptiles by water.

The washed sediment from the original ground coleoptiles was dried in air, ground through a Wiley mill (20-mesh screen), and dried to constant weight at a pressure of 0.03 mm Hg over phosphorus pentoxide. For convenience, this dried product (Fraction 4; 4.8286 g after removal of 0.0255 g for N determination; N, 1.58 % = 9.5 % protein) was regarded as the total primary cell wall material to which Fractions 5 to 9 are referred in terms of percent by dry weight (see discussion and table I).

Fraction 4 was extracted in a Soxhlet apparatus

[1] Received March 10, 1958.
[2] Issued as N.R.C. No. 4824.

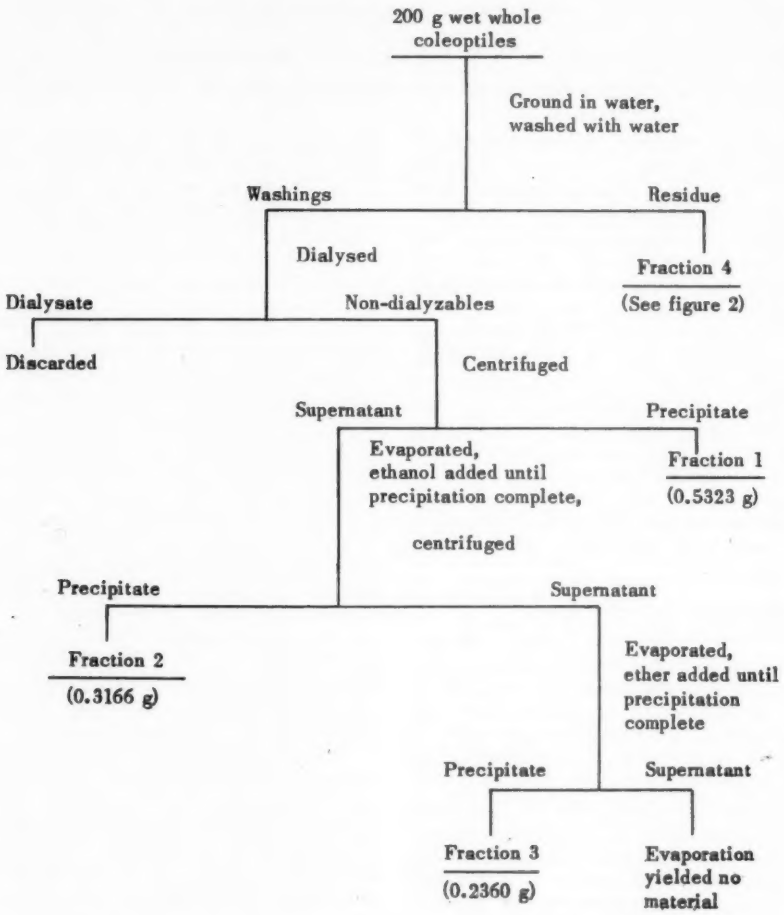

FIG. 1. Separation of polymeric material in the water washings of Avena coleoptiles.

with benzene : ethanol—2 : 1. Extractions were repeated until no more material was removed as determined by evaporating each extract to dryness. Four extractions were required, each one lasting 24 hours. The combined extracts were evaporated to give Fraction 5 (0.2008 g; 4.2 %). No carbohydrate material could be detected in this fraction by hydrolysis and chromatography. This fraction, which consisted of a crystalline and a waxy component, gave the following X-ray diffraction spacings in Å : 46, 7.8, broad band of scatter from 5.2 to 4.5, 4.25 (strong), 3.85 (strong), 3.05, 2.55 and 2.32.

The residue of Fraction 4 (4.6517 g) remaining after benzene : ethanol extraction was stirred with 70 ml of aqueous ammonium oxalate : oxalic acid (0.25 % of each) at 75° C for one hour (5). The mixture was centrifuged and the residue was repeatedly extracted in the same way until no precipitate was obtained when the supernatant liquor was diluted with 8 volumes of ethanol; four extractions were required. The combined extracts were diluted with two volumes of ethanol and the precipitate which formed was centrifuged and dried to give Fraction 6 (0.0859 g, 1.8 %). Hydrolysis of this fraction and chromatographic examination of the hydrolysate (solvents A and B) showed the presence of arabinose, xylose, glucose and a hexuronic acid. Fraction 6 was warmed gently in 15 ml of water and the mixture was centrifuged to remove sub-fraction 6 a. The supernatant liquor was diluted carefully with ethanol until a distinct precipitate formed which was centrifuged and classed as sub-fraction 6 b. When more ethanol was added to the supernatant liquor from sub-fraction 6 b a voluminous, gelatinous precipitate was formed. This precipitate was recovered by centrifuging and dried to give sub-fraction 6 c as a white powder.

Sub-fraction 6 a (0.0100 g, 0.2 %) was a dark brown powder, insoluble in water and yielding only traces of xylose, arabinose and glucose on hydrolysis. Sub-fraction 6 b was a white, water-soluble powder

(0.058 g, 1.2 %) having $[a]_D^{25} = +29° \pm 1°$ (C, 1.5 % in water), which gave a blue coloration with iodine-potassium iodide solution and on hydrolysis yielded mainly glucose with traces of arabinose and xylose as detected chromatographically. Sub-fraction 6 c (0.0165 g, 0.3 %) was a water-soluble powder showing $[a]_D^{25} = +220° \pm 4°$ (C, 0.4 % in water) and yielding on hydrolysis only a hexuronic acid detected by chromatography in solvents A, B and C. The hexuronic acid was chromatographically identified as galacturonic acid by specific spray reagents and by the fact that no lactone could be found on the chromatograms (7). Both glucuronic and mannuronic acids readily form lactones which run much faster than the parent acids on paper chromatograms. Galacturonic acid does not form a lactone. Identification of galacturonic acid was confirmed by simultaneous hydrolysis—oxidation (5) of a portion of fraction 6 c (0.010 g) by nitric acid (5 ml, specific gravity 1.15) to yield mucic acid with a melting point of 212 to 213° C (d).

The supernatant liquor from the precipitation of Fraction 6 was concentrated and ethanol was added

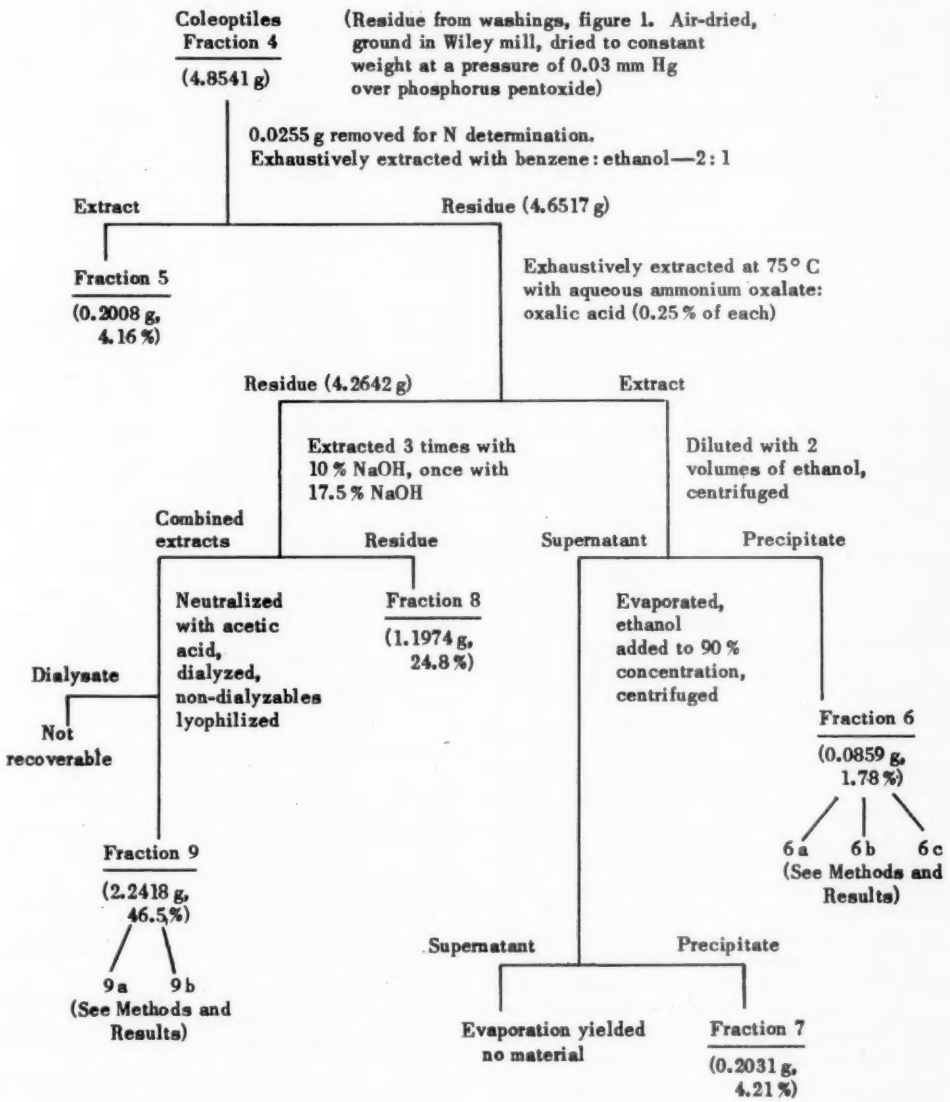
Coleoptiles
Fraction 4
(4.8541 g)
(Residue from washings, figure 1. Air-dried, ground in Wiley mill, dried to constant weight at a pressure of 0.03 mm Hg over phosphorus pentoxide)
0.0255 g removed for N determination.
Exhaustively extracted with benzene: ethanol—2: 1
Extract
Residue (4.6517 g)
Fraction 5
(0.2008 g, 4.16 %)
Exhaustively extracted at 75° C with aqueous ammonium oxalate: oxalic acid (0.25 % of each)
Residue (4.2642 g)
Extract
Extracted 3 times with 10 % NaOH, once with 17.5 % NaOH
Diluted with 2 volumes of ethanol, centrifuged
Combined extracts
Residue
Supernatant
Precipitate
Neutralized with acetic acid, dialyzed, non-dialyzables lyophilized
Fraction 8
(1.1974 g, 24.8 %)
Evaporated, ethanol added to 90 % concentration, centrifuged
Dialysate
Not recoverable
Fraction 6
(0.0859 g, 1.78 %)
6a 6b 6c
(See Methods and Results)
Fraction 9
(2.2418 g, 46.5 %)
9a 9b
(See Methods and Results)
Supernatant
Precipitate
Evaporation yielded no material
Fraction 7
(0.2031 g, 4.21 %)

FIG. 2. Fractionation of Avena coleoptile walls.

TABLE I

ANALYSIS OF AVENA COLEOPTILES

FRACTION	CLASS OF MATERIAL	PERCENTAGE OF DRY PRIMARY WALL	MONOSACCHARIDES RELEASED ON HYDROLYSIS				
			GALACTOSE	GLUCOSE	ARABINOSE	XYLOSE	HEXURONIC ACID
1, 2, 3	Water washings of coleoptiles Protein and carbohydrate	... ...	++ ++ +	+++ +++ +++	++ ++ ++	++ ++ ++	... ...
4	Washed sediment from ground coleoptiles (not hydrolyzed)	100	...	...	...	...	...
5	Fats, waxes, pigments	4.2	...	...	...	...	...
6 a	Polysaccharide	0.2	...	+	+	+	...
6 b	Starch-like polysaccharide	1.2	...	++++	+	+	...
6 c	Pectic substances	0.3	...	...	...	...	++++
7	Non-cellulosic polysaccharides	4.2	...	+	+++	+++	...
8	α-cellulose	24.8	see text				
9	Non-cellulosic polysaccharide	46.5	6.7 %	34.3 %	29.1 %	29.8 %	Trace
9 a	Water-soluble	About ⅔ Fraction 9	8.9 %	27.8 %	31.2 %	32.1 %	...
9 b	Water-insoluble	About ⅓ Fraction 9	4.5 %	41.0 %	27.0 %	27.6 %	...
	Protein	9.5					
	Total recovery	90.9					

to a concentration of 90 %. The precipitate which formed was centrifuged and dried giving Fraction 7 (0.2031 g; 4.2 %). Hydrolysis and chromatography (solvents A and C) of this fraction revealed the presence of arabinose, xylose and a trace of glucose. Evaporation of the supernatant liquor from this fraction yielded no further material.

The residue (4.2642 g) from the ammonium oxalate : oxalic acid extraction was shaken for three 18-hour periods with fresh portions of 70 ml of 10 % aqueous sodium hydroxide, and for one 18-hour period with 60 ml of 17.5 % aqueous sodium hydroxide. The residue was filtered with suction on a sintered glass funnel and washed thoroughly with water. It was then washed successively with 5 % aqueous acetic acid, water, 95 % ethanol, absolute ethanol, and ether before being dried as Fraction 8 (1.1974 g; 24.8 %; N, nil), the α-cellulose of Avena coleoptiles. The α-cellulose was completely hydrolyzed and the large excess of glucose in the hydrolysate was removed by fermentation with *Saccharomyces cerevisiae*, N.R.C. no. Y7 as described elsewhere (1). Chromatography (solvents A and C) of the glucose-free hydrolysate revealed the presence of galactose, mannose, arabinose, xylose and a trace of rhamnose.

The alkali extracts and washings, up to and including the 95 % ethanol washing, were combined and dialysed in Visking cellophane tubing against distilled water for four days, the water being changed twice a day. The non-dialyzable solution was then lyophilized to give Fraction 9 (2.2418 g; 46.5 %), the non-cellulosic polysaccharides of the cell wall. This fraction had $[\alpha]_D^{25} = -9.0° \pm 2°$ (C, 1.06% in *N* sodium hydroxide) and a number-average degree of polymerization of 16, determined by alkaline hypoiodite oxidation (13). On hydrolysis the polysaccharides yielded a mixture of sugars consisting of galactose, 6.7 %; glucose, 34.3 %; arabinose, 29.1 %; and xylose, 29.8 %. For this quantitative estimation the sugars were separated as bands on paper chromatograms (solvent A). The relative amounts of the separated sugars were then determined by measuring the area and intensity of their reaction with silver nitrate using a recording densitometer (14).

In a separate experiment the non-dialyzable material (Fraction 9) was separated, immediately after dialysis, into water-soluble and insoluble portions. The soluble portion (Fraction 9 a) represented two thirds by weight of Fraction 9, had $[\alpha]_D^{25} = -36.0° \pm 2°$ (C, 1.3 % in *N* sodium hydroxide), degree of polymerization 16, and on hydrolysis yielded a mixture of sugars consisting of galactose, 8.9 %; glucose, 27.8 %; arabinose, 31.2 %; and xylose 32.1 %. The insoluble portion (Fraction 9 b) constituted one third the weight of Fraction 9, had $[\alpha]_D^{25} = +50° \pm 2°$ (C, 0.5 % in *N* sodium hydroxide), degree of polymerization 8, and on hydrolysis yielded the following mixture of sugars: galactose, 4.5 %; glucose, 41.0 %; arabinose, 27.0 %; and xylose, 27.6 %.

The dialyzate of the alkali extract contained such large amounts (80 to 90 g) of sodium acetate that it was not feasible to recover any carbohydrate material which may have dialyzed.

To check recoveries in the fractionation, the residues from each extraction shown in figure 2 were recovered and dried. In this way it was found that recovery from the benzene : ethanol extraction, i.e., Fraction 5 plus residue, was 100.5 %. Recovery from the ammonium oxalate : oxalic acid extraction, Fractions 6 and 7 plus their residue, was 98 %. Recovery

in the alkali extraction was 81 % (Fractions 8 plus 9). If it is assumed that the protein (9.5 %) in the original dried wall fragments was lost by hydrolysis and dialysis during the alkali extraction, an assumption that was supported by the lack of nitrogen in Fraction 8, then the overall recovery in the fractionation was 90.9 %.

Complete Hydrolysis of Intact Avena Coleoptiles: To check for the presence of any large amounts of galacturonic acid which might have been lost during the detailed analysis just described, a sample (8.0 g) of wet, freshly harvested coleoptiles, equivalent to approximately 0.25 g dry weight, was completely hydrolyzed. The sample was dissolved in 20 ml of 72 % sulphuric acid and the solution was allowed to stand at room temperature for 1.5 hours. Water was then added until the acid concentration was 3 % and this mixture was refluxed for three hours. Acid was removed as barium sulphate and the salts were filtered and washed with hot water. The combined washings and filtrate were concentrated to about 25 ml and were passed over a column of Amberlite IR-120 exchange resin to remove barium. The column eluate was then concentrated and chromatographed on a full size sheet of Whatman no. 1 filter paper (solvent C). After guide strips had been cut and sprayed to locate the separated components, the hexuronic acid band was cut out and eluted with water, the eluate being evaporated to dryness giving a syrup (4.2 mg) having $[\alpha]_D^{23} = +57° \pm 5°$ (C, 0.42 % in water). This product was refluxed for 8 hours with 8 % methanolic hydrogen chloride (1 ml). The product was recovered by evaporating the solution and was reduced with sodium borohydride to the corresponding hexose glycoside. Sodium was removed from the reduction by Amberlite IR-120 exchange resin and borate was distilled off as its methanol complex. The residue from this reduction was hydrolyzed with *N* hydrochloric acid on a boiling water bath for 6 hours and the hydrolyzate was chromatographed (solvents A and C). Both galactose and glucose were found on the chromatograms in approximately equal proportions.

Discussion

The main results on which the discussion centres are summarized in table I.

The initial separation of ground coleoptiles into sediment and supernatant, designed to remove protoplasmic material from the walls, was only partially successful. The non-dialyzable carbohydrate material found in Fractions 1 to 3 of the supernatant (about 630 mg) almost certainly represented wall material since its monosaccharide composition closely resembled that of the major constituents of the wall. Non-dialyzable protein was divided roughly equally between sediment and supernatant with about 450 mg in each. On a percentage dry weight basis, the amount associated with the sediment (Fraction 4) was similar to that reported by Thimann and Bonner (20). However, it is uncertain how much of this was contaminating cytoplasm and how much can be regarded as true wall material. Undoubtedly a large proportion of this protein will be due to dense plasmodesmata which have been found frequently to penetrate even thick epidermal walls (4).

No detailed analysis was made of the fats, waxes and pigments (Fraction 5) extracted from the residue (Fraction 4) with benzene-ethanol, but it is interesting to note that the large X-ray spacing of 46 Å found in this fraction corresponds within experimental error to that of 44 Å detected previously in whole coleoptiles (2). Again, this differs significantly from the value of 60 Å reported earlier by Gunderman et al (9) for waxes extracted from Avena coleoptiles.

Removal of materials in Fraction 5 left the remaining tissue open to penetration by hydrophilic solvents. The ammonium oxalate : oxalic acid extraction was designed to remove pectic substances. In recent years it has been shown that ammonium oxalate : oxalic acid solution is a more efficient and selective extracting medium for pectic materials than ammonium oxalate solution alone (5). According to the latest rules of nomenclature "pectic substances" are defined as "those complex, colloidal carbohydrate derivatives which . . . contain a large proportion of anhydrogalacturonic acid units . . . . The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases" (23, p. 162). When applied to coleoptiles the ammonium oxalate : oxalic acid extraction removed a mixture of polysaccharides which were separated by a sub-fractionation with ethanol-water. By this procedure the pectic material (Fraction 6 c) was separated from a starch-like polysaccharide containing largely glucose, which gave a blue coloration with iodine (Fraction 6 b) and from non-cellulosic polysaccharides (Fractions 6 a and 7) containing arabinose and xylose. The high positive optical rotation of +220° (cf. 23, p. 185) and the identification of galacturonic acid as the only product of hydrolysis characterized Fraction 6 c as pectic material. The absence of hexuronic acid in hydrolysates of the other fractions, except for a trace in Fraction 9, indicated that the small amount of pectic material in the coleoptiles was removed in this extraction.

The low pectic content of these walls was confirmed by complete hydrolysis of intact coleoptiles, the results of which showed that all hexuronic acids represent only about 1.6 % of the total dry weight of coleoptiles and this is composed of roughly equal quantities of glucuronic and galacturonic acids. This total quantity of hexuronic acids which undoubtedly includes some soluble sugars corresponds sufficiently closely with what was detected in Fractions 6 c and 9 of the detailed analysis to indicate that no large quantity of pectic material (polygalacturonic acid) could have been lost.

By far the largest single class of materials in the coleoptiles were the non-cellulosic polysaccharides, usually termed hemicelluloses, extracted by alkali. These were composed of the monosaccharide units galactose, glucose, arabinose and xylose and the differ-

ences in optical rotation and monosaccharide composition of the subfractions indicated that the material was a mixture of several polysaccharide species.

The alkali extraction and subsequent isolation of α-cellulose and non-cellulosic polysaccharides was the only step in the analysis where loss of material occurred. Part of this was caused by alkaline hydrolysis of the 9.5 % protein in the coleoptiles and its loss as amino acids during dialysis. The remaining loss was probably caused by dialysis of short-chain polysaccharide material having the same composition as the polysaccharide material that was recovered. The non-cellulosic polysaccharides that were recovered (Fraction 9) were of quite short chain length (degree of polymerization, 8 to 16) and it is possible that polysaccharides with a degree of polymerization of less than 8 would pass through the dialysis membrane and be lost. The very large amounts of sodium acetate in this dialyzate precluded any attempts to check this point by recovering the carbohydrate material from the dialyzate. It is also possible that the strong alkali used in the extraction caused degradation of some of the non-cellulosic polysaccharides to fragments sufficiently small to be lost on dialysis (22).

The remainder of the wall consisted of α-cellulose (Fraction 8) which was present in a concentration (25 %) generally lower than that reported for other primary walls (6, p. 134). Traces of monosaccharide residues other than glucose were present in the α-cellulose. Such residues are commonly found in α-cellulose and their significance has been discussed previously (1, 3).

The picture this analysis gives of the chemical composition of primary walls of Avena coleoptiles is comparatively simple. The walls consist principally of the relatively inert substances, cellulose and non-cellulosic polysaccharides. The latter materials, although they may be numerous and may differ in detailed structure, appear to be composed almost entirely of similar residues.

The most important findings in the study concerned the pectic content, which was considerably lower than that reported in other analyses of primary walls (6, 21). It is probable that the primary walls of different tissues or species vary markedly in pectic content. However, the sole evidence for the presence of pectin has often been the ability of ammonium oxalate to extract material from the wall. The present results show that significant quantities of polysaccharides other than pectin, including starch-like material, can be extracted with this solvent.

More significantly, however, these results show that an appreciable proportion of pectic material is not in fact a prerequisite for rapidly extending walls. Because of its ability to form gels, pectin has usually been vested with the role of supplying flexibility to cell walls. Kerr (12) has described it as the "continuous phase" of the wall, the "discontinuous phase" being provided by the cellulose skeleton. Furthermore, recent results from studies on Avena coleoptiles (15, 16, 19) have suggested that the way in which auxin plasticizes walls to permit increased elongation of cells is by affecting the degree of methyl esterification of the pectic materials in the walls. Other authors (8) have inferred from effects of auxin on the binding of pectin methylesterase in the wall that in promoting elongation, auxin acts through pectin. However, it is difficult to imagine how the minute pectic content found here could have significant influence on the physical properties of the walls: hexuronic acid residues, either as pectic substances or as components in non-cellulosic polysaccharide chains, could possibly have a disproportionate effect in plasticizing the wall by occupying strategic positions in the wall structure, but the degree of economy and order in the distribution of these residues required to achieve such a system would appear to be so high as to make it improbable.

If the non-cellulosic portion of the primary wall is to be considered an active agent in wall structure, it seems that attention must be turned to the preponderant non-cellulosic polysaccharides. These compounds do not have the obvious capacity for reaction which could be ascribed to the ester groups of pectin. However, the most fruitful approach to a better understanding of the physiological aspects of the structure and metabolism of primary cell walls is probably a more thorough study of these substances.

Summary

The primary walls of Avena coleoptiles were fractionated chemically and the constituent monosaccharides of the caroohydrate components were identified by paper chromatography and other methods. On a dry weight basis, at least 51 % of the walls was a mixture of hemicelluloses containing principally residues of galactose, glucose, arabinose and xylose; α-cellulose represented 25 %, fats, waxes and pigments 4 % and protein 9 %. The fraction obtained by ammonium oxalate : oxalic acid extraction was composed largely of a starch-like polysaccharide (1.2 % of the dry walls); only a small amount of pectic substances was obtained representing 0.3 % of the dry walls. The very low pectic content of the walls was confirmed by the finding of less than 1 % galacturonic acid on a dry weight basis in a hydrolysate of fresh coleoptiles.

The results suggest that the physical properties of these elongating walls depend more on the preponderant hemicelluloses than on the negligible quantity of pectic substances.

The authors are grateful to Mr. A. D. Gordon for technical assistance.

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EFFECT OF LIGHT ON THE CO_2 ABSORPTION AND EVOLUTION BY KALANCHOË, WHEAT AND PEA LEAVES[1,2]

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A common technique for the determination of the true photosynthetic rate is to measure the apparent CO_2 intake in light and then add to this the amount of CO_2 respired by comparable material in darkness. The validity of this technique is based on the assumption that light does not affect respiration.

Within the last years support of this assumption has come from several experiments. Davis observed that light had no effect on the consumption of oxygen by a Chlorella mutant, which had lost its ability to carry on photosynthesis (3). In a series of experiments with oxygen isotopes, Brown has shown that when leaves of several higher plants or Chlorella were illuminated in air enriched with O^{18}, their consumption of heavy oxygen was unaffected by light (1, 5). Photoinhibition at higher light intensities was observed, however, for several algae (5). In Anabaena, photoinhibition was observed at oxygen concentration below 0.5 % in the gas phase, while photostimulation was apparent at higher light intensities (2).

In all these experiments, however, respiration was measured by oxygen consumption and it is desirable to inquire whether the same results are obtained when respiration is measured by CO_2 production.

[1] Received revised manuscript March 15, 1958.
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Some evidence indicates that the results are not identical.

In starved cells of *Ochromonas malhamensis*, O_2^{18} consumption was twice as high in light as in the dark, though no increase in carbon dioxide production could be observed using $C^{13}O_2$ (12).

Weigl et al (11) placed barley plants in a mixture of $C^{12}O_2$ plus $C^{14}O_2$ and followed changes in these two gases in light and in darkness. On illumination they observed a decrease in the specific activity of the $C^{14}O_2$ around the plants. This shows that the photosynthetic readsorption of respiratory $C^{12}O_2$ is by no means complete, and some of it is evolved. These workers concluded that "the mean rate of respiratory CO_2 evolution in strong light was found to be less than that in the dark." Such a conclusion, however, was reached from the kinetic analysis of the data obtained from a single experiment only. Moreover, as they say, "the rate of respiration was by no means constant over the period of illumination; initially, at high CO_2 concentrations, it appeared to be even faster than dark respiration, whereas it dropped well below half the dark rate at subsequent low CO_2 pressures." Under these conditions one can hardly draw a clear-cut conclusion on whether CO_2 evolution in light was greater or less than in the dark.

A recent study by Decker (4), using a closed system containing an infra-red CO_2 analyzer led to the conclusion that, in all of the 9 species of higher plants he investigated, CO_2 output was greater in the light than in darkness.

Nielsen (8) permitted Chlorella cells to carry on photosynthesis for two hours in $C^{14}O_2$ and then placed them in darkness for 1.5 hours. At the end of this time cells were divided into two samples. One was placed in light, the other in darkness, and production of $C^{14}O_2$ from each sample was measured for five hours. It was found that light suppressed production of $C^{14}O_2$ by cells to a value about one third of that in darkness. A similar light suppression of $C^{14}O_2$ was observed by this worker also in Myriophyllum. Such results could be explained either as due to the depressing effect of light on CO_2 production or to the partial re-utilization of the respired CO_2 in photosynthesis. Working with a marine flagellate *Dunaliella euchlora* Ryther (9) concluded that in this organism $C^{14}O_2$ respired was completely re-assimilated during photosynthesis.

According to the conclusion reached by Weigl et al (11), recent products of photosynthesis are transformed during illumination into some substances, which are not available as a respiratory substrate in light, though they are available in darkness. It is possible, therefore, that the photoinhibition in the evolution of recently assimilated $C^{14}O_2$ observed by Nielsen is due to this fact and not to the inhibition of respiration by light.

The work reported below was undertaken to investigate more fully the effect of light on the evolution of carbon dioxide from the leaves of higher plants using $C^{14}O_2$.

Materials and Methods

Single clone plants of *Kalanchoë blossfeldiana* Poellnitz var. Tom Thumb, about 8 months old were maintained for about 6 weeks on either a 16- or 8-hour day in constant light and temperature chambers. These chambers, located at Harvard University, have been described elsewhere (6). Wheat (*Triticum vulgare* Vill.), var. Thatcher, and peas (*Pisum sativum* L.), var. Laxton's Progress, were germinated and grown in flats in the greenhouse during the month of February. Natural sunlight was supplemented by illumination provided by several banks of fluorescent lights. The total period of illumination was equal to a 16-hour day. The leaves of Kalanchoë were taken from the fully developed plants, while the wheat seedlings were 14 to 16 days old, and the pea plants 22 days old. For each experiment, leaves were detached directly after the dark period or after five hours of photosynthesis in artificial light (350 ft-c). In this manner leaves received either dark or light pretreatment, prior to the experimental period.

Detached leaves were placed on water in a special glass photosynthetic chamber. Sufficient CO_2 to bring the concentration up to 5 % was released with 1 *N* H_2SO_4 from 5 ml of $Na_2C^{14}O_3$ solution containing 10 to 100 μc of C^{14}, and the leaves were left either in darkness or in light for one hour. Illumination was provided by several incandescent bulbs filtered through a water filter, the intensity being about 800 ft-c. The temperature within the photosynthetic chamber during the experiments was $20 \pm 2°$ C.

At the end of each experiment the residual CO_2 was removed from the chamber by evacuating it three times, and was absorbed in 1 *N* NaOH. The total CO_2 present in the photosynthetic chamber at the end of the experiment was determined by titration of the standard alkali, and its total activity by plating and counting the precipitated samples of Ba $C^{14}O_3$.

Prior to each experiment, two control runs were carried out, in which the procedure outlined above was followed, with the exception that no plant material was present in the chamber. These two controls provided measures of the absolute amount, ac-

Table I

Intake and Evolution of CO_2 by Kalanchoë Leaves (Mg of CO_2/hr × g fresh weight)

	Pre-treatment	Experimental conditions	$C^{14}O_2$ uptake	$C^{12}O_2$ output
Short day plants, 8 months old	Dark	Dark	0.85 ± 0.24	0.65 ± 0.29
	5 Hrs light	"	1.07 ± 0.26	1.78 ± 0.31
	Dark	Light	0.58 ± 0.23	1.03 ± 0.27
	5 Hrs light	"	0.32 ± 0.23	0.76 ± 0.28
Long day plants, 8 months old	Dark	Dark	1.20 ± 0.27	1.03 ± 0.31
	5 Hrs light	"	1.32 ± 0.26	0.62 ± 0.32
	Dark	Light	1.45 ± 0.28	0.57 ± 0.31
	5 Hrs light	"	1.25 ± 0.29	0.27 ± 0.36

tivity and hence specific activity of the $C^{14}O_2$ offered to leaf material in subsequent experiments, and made possible an assessment of the errors involved.

From the data so obtained it was possible to calculate the weight in milligrams of $C^{14}O_2$ absorbed and of $C^{12}O_2$ evolved by the leaves. A sample calculation is shown below:

	$C^{14}O_2$ extracted from the chamber	
	Mg, determined by titration	Activity in 10^6 cpm units determined by counting (all radioactive samples were counted to less than 2 % statistical error)
Control 1	24.6	98.0
Control 2	24.0	103.2
Mean	24.3 ± 0.3 *	100.6 ± 2.6 *
Experiment	16.6 ± 0.3 *	56.9 ± 2.1 **

∴ Specific activity of $C^{14}O_2$ offered =

$$\frac{100.6 \pm 2.6}{24.3 \pm 0.3} = 4.14 \pm 0.12 \text{ cpm } (\times 10^6)/\text{mg } \dagger$$

Total activity offered 100.6 ± 2.6 cpm ($\times 10^6$)
Activity remaining after 1 hour 56.9 ± 2.1 cpm ($\times 10^6$)

∴ Activity taken up by leaf = 43.6 ± 3.3 cpm ($\times 10^6$) ††

$$\text{Wt of } C^{14}O_2 \text{ taken up by leaf} = \frac{\text{Activity taken up by leaf}}{\text{Specific activity}}$$

$$= \frac{43.6 \pm 3.3}{4.14 \pm 0.12} = 10.5 \pm (0.9) \text{ mg } \dagger$$

Wt of CO_2 offered, mg 24.3 ± 0.3
Wt remaining after 1 hour, mg 16.6 ± 0.3

∴ Net uptake of CO_2, mg = 7.7 ± 0.4 ††

but, uptake of $C^{14}O_2$ = 10.5 ± 0.9 mg
and, net uptake of CO_2 = 7.7 ± 0.4 mg

∴ Output of $C^{12}O_2$ = 2.8 ± 0.9 mg ††

* Probable error (r_a) of the mean (a), given by:

$$r = k\sqrt{\frac{d_a^2}{n(n-1)}} \quad \text{where } k = 1 \text{ for } n = 2 \text{ (7)}$$

** Probable error (r) of a single observation, calculated by proportionality to the probable error (single observation) of the mean of the controls, i.e.

$$\frac{16.6}{24.3} r, \text{ where } r = lx\sqrt{\frac{\Sigma d_a^2}{n-1}} = r_a\sqrt{n}$$

† Probable error of a quotient, given by

$$r_z = \frac{l}{y}\sqrt{(r_x)^2 + (z \cdot r_y)^2}, \text{ where } z \pm r_z = \frac{x \pm r_x}{y \pm r_y}$$

†† Probable error of a difference, given by

$$r_z = \sqrt{(r_x)^2 + (r_y)^2},$$
$$\text{where } z \pm r_z = (x \pm r_x) - (y \pm r_y)$$

The values presented in tables I, II and III were obtained by dividing the weights of CO_2 together with their associated errors by the fresh weight of leaf material used.

Results and Discussion

Tables I to III show that in nearly all the instances studied, some CO_2 is taken up in the dark and some is evolved in the light. The extent of the CO_2 exchange is different in the different plants. In wheat (table II), CO_2 uptake is much greater in light than in the dark, as might be expected on traditional grounds, and the same is true in the pea (table III), where dark uptake is virtually absent. In Kalanchoë, however (table I), the dark uptake is quite large, averaging 1.1 mg CO_2 per hour per gram of leaf. Although it depends somewhat on the pretreatment, in general its magnitude in Kalanchoë is about the same as in the light, for plants on long day, and actually greater in the dark for plants on short day. The phenomenon of dark uptake of CO_2 by succulent leaves is, of course, well known. Summarizing, we may say that light greatly promotes CO_2 uptake in wheat and in the pea, does not much affect it in long day Kalanchoë, and depresses it in Kalanchoë on short day.

As to the CO_2 output, light on the whole acts to depress it in Kalanchoë, except in the dark pretreated short day plants (table I). In wheat (table II) light roughly doubles the CO_2 output, while in the pea (table III), on the other hand, light reduces it by some 75 %. Thus only in wheat is the behavior like that reported by Decker (4).

The data on wheat might be compared with those of Sen and Leopold (10) on barley, which also showed that the dark CO_2 uptake was a function of the preceding light treatment. However, they state that dark uptake decreases the longer the time after the end of the light period, which does not appear to

Table II

Intake and Evolution of CO_2 by Primary Wheat Leaves
(Mg of CO_2/hr × g fresh weight)

	Pretreatment	Experimental conditions	$C^{14}O_2$ uptake	$C^{12}O_2$ output
Expt 1	Dark	Dark	0.21 ± 0.36	0.52 ± 0.40
Plants	5 Hrs light	"	0.06 ± 0.37	0.06 ± 0.41
16 days	Dark	Light	4.01 ± 0.32	1.07 ± 0.36
old	5 Hrs light	"	3.73 ± 0.29	0.95 ± 0.33
Expt. 2	Dark	Dark	0.49 ± 0.28	0.24 ± 0.33
Plants	5 Hrs light	"	0.57 ± 0.23	0.64 ± 0.29
14 days	Dark	Light	4.62 ± 0.19	1.68 ± 0.22
old	5 Hrs light	"	4.40 ± 0.18	1.67 ± 0.20

Table III

Intake and Evolution of CO_2 by Pea Leaves
(Mg of CO_2/hr × g fresh weight)

	Pretreatment	Experimental conditions	$C^{14}O_2$ uptake	$C^{12}O_2$ output
Plants	Dark	Dark	0.01 ± 0.31	2.73 ± 0.36
22 days	5 Hrs light	"	0 ± 0.28	2.26 ± 0.31
old	Dark	Light	3.69 ± 0.28	0.69 ± 0.30
	5 Hrs light	"	3.83 ± 0.27	0.77 ± 0.29

be always the case in wheat (table II). These workers also noted an important change in the amount of CO_2 taken up in the light when the plants had previously been given photo-inductive treatment.

The findings with Kalanchoë can be compared with the earlier measurements of overall CO_2 exchange (6). The striking observation of a net CO_2 evolution in light taking place in plants which have been kept on short day but not in those on long day is confirmed in table I. However, the behavior of the plants in the dark was equivocal and a detailed study of the dark exchange as a function of time after illumination would be necessary to establish a correlation with the overall exchange data.

The data give no indication as to whether the CO_2 absorbed is taken up by buffer systems or represents actual carboxylation. However, absorption of CO_2 by buffers would hardly be expected to be sensitive to light. Furthermore, since carboxylation is known to occur in succulents, it may well take place to a smaller extent in wheat.

The results as a whole certainly indicate that CO_2 moves freely into and out of leaves, irrespective of whether they are in light or darkness. In all three plants studied, some CO_2 was evolved in light. In light-pretreated Kalanchoë and in the pea, this evolution of CO_2 in the light was less than that in the dark, but in wheat the reverse was true, and light actually stimulated CO_2 output in all the four instances.

Since during illumination there is a considerable movement of CO_2 from the outside into the leaf, it is perhaps surprising that some CO_2 can simultaneously move out of the leaf, with its complement of photosynthesizing chloroplasts. While the present data provide no explanation for the evolution of CO_2 in light, several tentative suggestions can be made:

a. Some additional respiratory substrate may become available to the cell in the light, which is not available in darkness. The observed CO_2 evolution in light may represent, therefore, an "additional respiration."

b. There is an increasing body of evidence that the metabolic machinery of the cell is strictly localized. It is possible that one area of the cell periphery serves for the movement of CO_2 outwards and a different one for its movement inwards.

c. Anatomical characteristics of the photosynthesizing organ may be of importance. For example, mesophyll cells, which are full of chloroplasts, may be responsible for the intake of CO_2 into a leaf, while epidermal cells, which have virtually no chloroplasts, may be responsible for the evolution of CO_2 in light.

Whatever the explanation, it is clear that all three plants evolved CO_2 while illuminated. Furthermore, in wheat, the output in the light was greater than in darkness. A similar conclusion for the initial CO_2 output on illumination was drawn by Decker for 9 other higher plant species.

Summary

1. Detached leaves of Kalanchoë, wheat and pea were placed in a glass chamber in the presence of known amounts of $C^{14}O_2$ and were kept in either darkness or light for one hour. The residual $C^{14}O_2$ was then removed and its amount and activity determined. From these analyses the amounts of $C^{14}O_2$ taken in and $C^{12}O_2$ evolved by the leaves were calculated.

2. In Kalanchoë and wheat, CO_2 uptake was observed both in light and in darkness, while in the pea it could be demonstrated only in the light.

3. Whether or not photosynthetic CO_2 consumption prevailed, in each species examined CO_2 evolution also was observed. In wheat CO_2 evolution was enhanced by illumination while in Kalanchoë and peas it usually was reduced.

4. It is concluded, therefore, that addition of the amount of CO_2 respired in darkness to that absorbed in light will not necessarily produce the value for the true photosynthesis of a leaf.

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CIRCULATION PATTERNS FOR PHOSPHORUS, SULFUR AND CALCIUM IN THE BEAN PLANT[1,2]

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Perhaps the earliest suggestion that soluble materials may make a circuit within a plant was made by Hartig (16). He concluded that materials assimilated in the leaves moved downward in the bark and were stored in the parenchyma and rays. In the spring these materials were brought into solution, passed into the trachea and ascended with the moving current of water. Atkins (1) expressed essentially the same views, and later the data of Mason and Maskell (20) indicated that such a circuit might be made without an intervening storage period. The latter investigators suggested that nitrogen, phosphorus, potassium, and other minerals ascended the stem primarily in the xylem, and any excess not currently used in the leaves was re-exported downward in the phloem. The ratios of N, P, and K to the carbohydrate moving downward in the phloem appeared to be in excess of that required for growth of the roots, so it was suggested that the excess was liberated into the xylem sap to re-ascend the stem. Biddulph (2) showed the rapidity of movement of phosphorus in the phloem of the bean plant and the possibility of a more or less continual circulation of this element throughout the plant. Helder (17) has confirmed the circulation of phosphorus in barley and bean plants. A free circulation of phosphorus is to be expected on the basis of its mobility in the phloem and its rapid metabolic turnover in many cellular reactions which serve to maintain a supply of inorganic phosphate in the cellular fluids.

Prior to the work of Thomas et al (28) sulfur was considered to be relatively immobile in plants (Wood (29)), but the demonstration by Thomas et al of movement from the leaves on one stem of a multi-stemmed alfalfa plant downward to the crown and then upward in another stem, left little doubt of its mobility. Biddulph et al (5) showed sulfur to be freely mobile in the phloem of the bean plant, moving at rates comparable to other mobile substances. It is suspected that the earlier views of immobility may stem from the rapidity with which sulfur may be metabolically incorporated into newly formed protein, leaving little free to re-circulate. The present investigation is intended to test this concept.

Calcium has been considered immobile in the phloem since the early investigations of the withdrawal of minerals from leaves prior to their abscission in the autumn (22). Bledsoe et al (9) recently provided a very direct demonstration of the immobility of calcium in the phloem of the peanut plant, and subsequently the immobility of calcium-45 in the phloem has been confirmed a number of times (3, 27, 10).

The objective of the present study is to compare, by means of a direct method, the circulation patterns of three mineral nutrients which are suspected of displaying distinctive patterns of mobility within the plant. For this study the Red Kidney bean plant, grown under carefully controlled experimental conditions, was selected. The methods which might be selected for such a study include the following: (A) the upward movement of a tracer from root to leaves in one set of plants and the downward movement from leaf to roots in another; (B) the determination of the amount of a tracer substance in various plant parts at definite intervals following application of the tracer to the roots; and (C) a radioautographic study of the fate of a single dose of radioactive tracer at successive intervals following its application to the root system. The latter method (C) is employed in the present study. It differs from (B) only in the method employed for detecting the radioactivity.

Millikan (23) has shown that the radioautographic method may yield unreliable results if the tracer-containing plants are dried slowly prior to their exposure to the film. The tracer migrated from interveinal tissue to the veins during the drying process. Pallas and Crafts (24) have shown that during the drying process there was a migration of 2,4-D applied to leaves when the plants were frozen prior to drying.

Our experience is that rapid drying in hot blotters in a forced draft oven is a reliable procedure for depicting phloem limited materials if the plants have not been mechanically injured. In such plants no significant shifting of radioactive materials within the tissue has been observed. However, if the plants are injured by steaming, freezing, or cutting, thereby facilitating transfer to the xylem, there will result in the drying process very pronounced distortions of the initial distribution pattern of the radioactive materials. A single injury to the conductive tissues of the leaf, such as was used earlier in the leaf flap method of introducing tracer (2), appears to yield reliable results as long as a second injury is not made to the conductive tissue in some other part of the plant.

METHODS

Red Kidney bean plants were used as experimental material. They were grown in one half strength Hoagland solution for a period of 8 days from the straightening of the hypocotyl, in the case of the plants to receive S-35, and for 10 days for those to receive P-32 or Ca-45. Growth conditions were $23 \pm 1°$ C, $60 \pm 5\%$ RH and 1,000 to 1,200 ft-c of fluorescent light on a 12-hour photoperiod.

[1] Received April 2, 1958.

[2] This investigation was supported in part by U.S. A.E.C., Div. of Biol. and Med. Contract No. AT(45-1)-213.

[3] The radioisotopes were acquired from the U.S.A.E.C., Oak Ridge, Tenn.

Younger plants were used with S-35 because mobility of this element had been shown in older plants (5), and younger plants were considered to provide more critical material for the demonstration of mobility as metabolic capture would be relatively greater.

At 8:45 A.M. on the treatment day the radioactive isotope was placed in the nutrient solutions, i.e., approximately 88, 88 and 10 μc/L for P-32, Ca-45 and S-35, respectively. At 9:45 A.M. (1 hr later) all plants were returned to separate normal (non-radioactive) solutions, the roots being dipped once in distilled water in transit. At subsequent intervals of 0, 6, 12, 24, 48, and 96 hours, plants were removed and quickly dried between blotters in a forced draft oven. Radioautograms were then prepared of all plants. The members of each group, i.e., a, b, c, d, e, and f, corresponding to migration periods of 0, 6, 12, 24, 48 and 96 hours, respectively, were all given the same length of exposure to the films, so that the relative densities of the radioautograms permit, when properly interpreted, an estimation of the concentration of the radioisotope in the tissue.

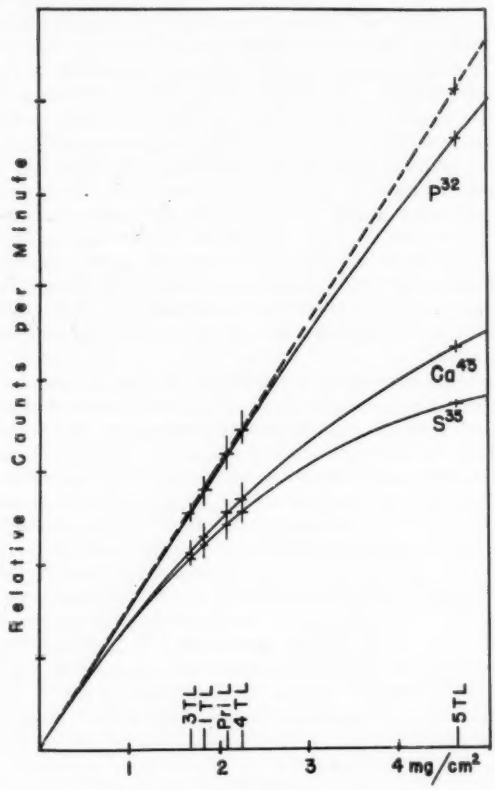

FIG. 1. Experimentally determined self absorption curves for P-32, Ca-45 and S-35. The ordinates have been adjusted so that the approximate self absorption factors can be read from the composited curves. For the 3rd, 4th, and 5th trifoliate leaves (TL) the approximate correction factors are, respectively: P-32, 1.00, 1.00, 1.06; Ca-45, 1.15, 1.25, 1.64; S-35, 1.20, 1.31, 1.89. Factors for the 2nd TL corresponded to those for the 1st TL.

Proper interpretation of the radioautograms depends on a knowledge of the self absorption characteristics of the isotopes used and a measure of the mass per unit area of the tissues involved. Figure 1 shows the self absorption curves for P-32, S-35, and Ca-45, together with the mass per unit area of the dried leaves of a representative bean plant. Correction factors are significant for Ca-45 and S-35, especially in the case of the younger leaves. Here the density rendition of the image of the young leaves near the stem tip falls significantly short of a linear relationship with radioactivity because of self absorption. It should also be understood that in dealing with radioautograms, as with any photographic rendition, the density of the image produced is proportional to the logarithm of the exposure and not to the exposure as such.

In summary one may safely disregard self absorption corrections for the P-32 radioautograms for all leaves but the youngest. Here the actual radioactivity will be slightly underrecorded because of self absorption. In the case of the S-35 radioautograms, the youngest leaves will be significantly underrecorded, but since interpretation of these radioautograms is largely dependent on a comparison between the primary leaf and 1st and 2nd trifoliate leaf activities, all of which have quite similar self absorption characteristics, corrections here are relatively unimportant. And, finally, in the case of the Ca-45 radioautograms, the radiation from the younger leaves will also be underrecorded, but their activity is so very slight in comparison with activities in the older leaves that self absorption corrections are trivial by comparison and need only minor attention in properly interpreting the results.

RESULTS

The results of this study are presented as a series of radioautograms representing the progressive distribution, from zero to 96 hours, of the radioisotopes administered during the one-hour period. The radioautograms of P-32, S-35, and Ca-45 are shown in figures 2, 3, and 4, respectively, and the normal distribution of the elements in the various plant parts, as related to the concentration in the nutrient media, in figure 5. An examination of the radioautograms of figure 2, representing P-32, shows that a significant portion of the labeled phosphorus which entered the plants remained sufficiently mobile to supply the continuously growing young leaves and stem apexes, where the highest concentration of radioisotope in the aerial parts accumulated, for the whole of the 96-hour period. The mature primary leaves show their peak concentration of tracer at the close of the 6-hour period, the delay being due to a relatively slow movement of phosphorus into the transpiration stream (compare S-35 movement into the primary leaves, fig 3 a). A subsequent withdrawal of P-32

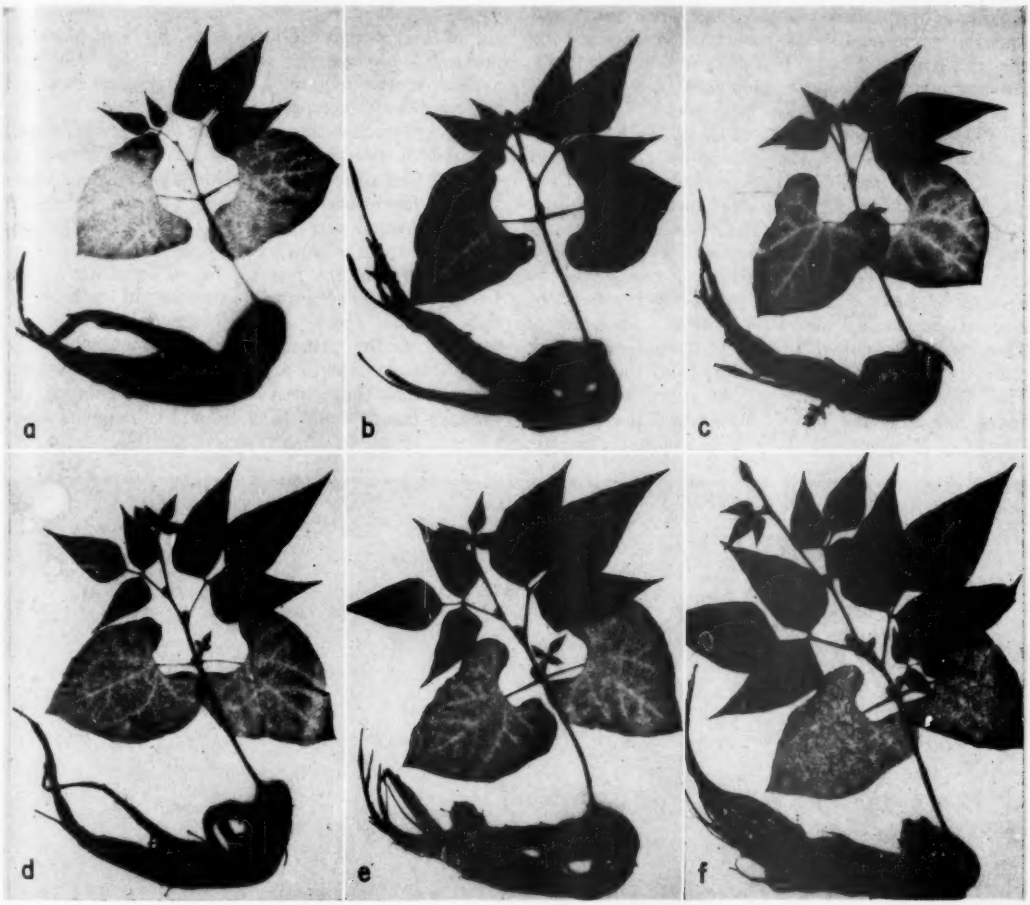

FIG. 2. The progressive distribution through a 96-hour period of a single aliquot (88 μc/L/plant) of P-32 administered via the nutrient solution during a 1-hour period. Exposure of dried plant to film = 17 days. The time sequence, in hours, was: a = 0, b = 6, c = 12, d = 24, e = 48, and f = 96.

from the primary leaves indicated that they were the source of a portion of the tracer which moved into the stem apexes as they continued to elongate.

Dilution of the initial aliquot of radioisotope acquired by the various plant parts is brought about by the subsequent growth of the tissues; the amount of dilution being proportional to the amount of growth made. Therefore, a high concentration of the tracer can be maintained in growing tissue only by importation. In the instance of the P-32-treated plants the importation was adequate to maintain a high concentration throughout the whole of the 96-hour period.

All plants showed a marked retention, within the root system, of a large portion of the P-32 acquired from the nutrient medium. This is adequately displayed in figure 2 where it is evident that P-32 was retained for the entire period (96 hrs). It is possible that a portion of the radioisotope which belatedly accumulated in the younger growing regions of the plant was derived from this source by a slow turnover into the transpiration stream. But if there were no subsequent removal, or turnover of the ascending P-32, particularly in mature leaves, those tissues with the longest history of accumulation, i.e., the primary leaves, would be highest in tracer. The fact that the older primary leaves did not progressively accumulate tracer indicated that, if the root did release tracer, it moved both into and out of the primary leaves, for it is not probable that upward moving tracer would enter these leaves during one hour and refrain from entering at another.

A comparison of the P-32 distribution (fig 2), with the total phosphorus concentration (fig 5), shows that as near as can be judged the P-32 is distributed, by the close of the 6-hour period, in the same ap-

proximate proportions as the total phosphorus and that this same relative distribution is maintained for the remaining 90-hour period. Within a rapidly growing plant this can be possible only by means of a continual redistribution of the tracer. (Compare with the distribution pattern of Ca-45, fig 4.)

The distribution pattern for S-35 differed from that of P-32 in that sulfur, while freely mobile in the plant, was more quickly captured in metabolic processes which immobilized it. The metabolic capture of the tracer sulfur took place in those leaves displaying a rapid growth rate. The primary leaves served as a source of some of the tracer sulfur which ultimately reached the young leaves as is evident from figure 3. This removal resulted in the ultimate and rather complete depletion of tracer sulfur from these leaves. The depletion occurred within approximately 12 hours following the initial delivery of the S-35 to them, and they remained depleted until the end of the 96-hour period. The roots of the bean plant are normally comparatively low in sulfur (fig 5) so that a possible slow release of the tracer by the roots did not complicate interpretation of the results.

By comparing the initial (fig 3 a) and the final (fig 3 f) distribution of S-35 in the plant, it becomes obvious that most of the "resident" sulfur in mature primary leaves was acquired early, that is, before the S-35 absorption period, and that relatively little subsequently acquired sulfur was retained. The absence of the tracer in the primary leaves following an initial delivery to them, and its presence in the tissue of stem tip and roots produced subsequent to its absorption, is the principal evidence upon which the statement of mobility and rapid metabolic capture is based. Had there been a sustained circulation the primary leaves would have, at any subsequent inter-

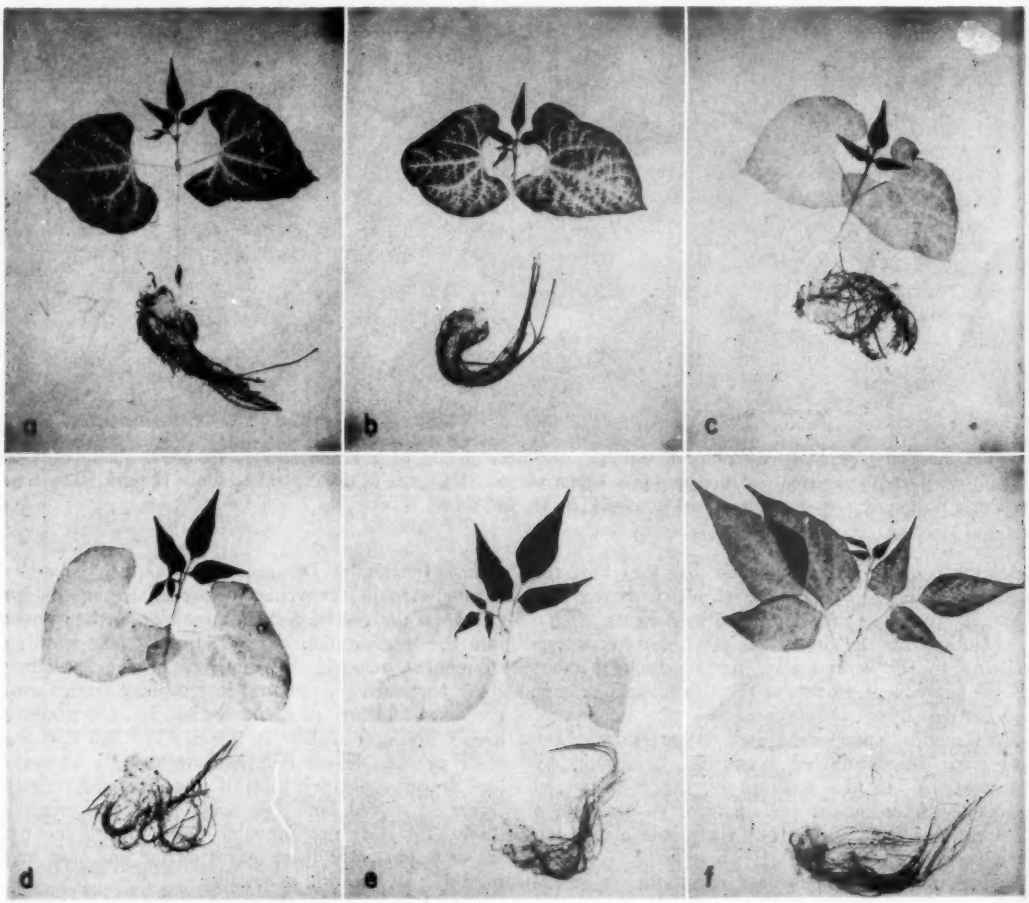

FIG. 3. The progressive distribution through a 96-hour period of a single aliquot (10 μc/L/plant) of S-35 administered via the nutrient solution during a 1-hour period. Exposure of dried plant to film = 190 days. The time sequence, in hours, was: a = 0, b = 6, c = 12, d = 24, e = 48, and f = 96.

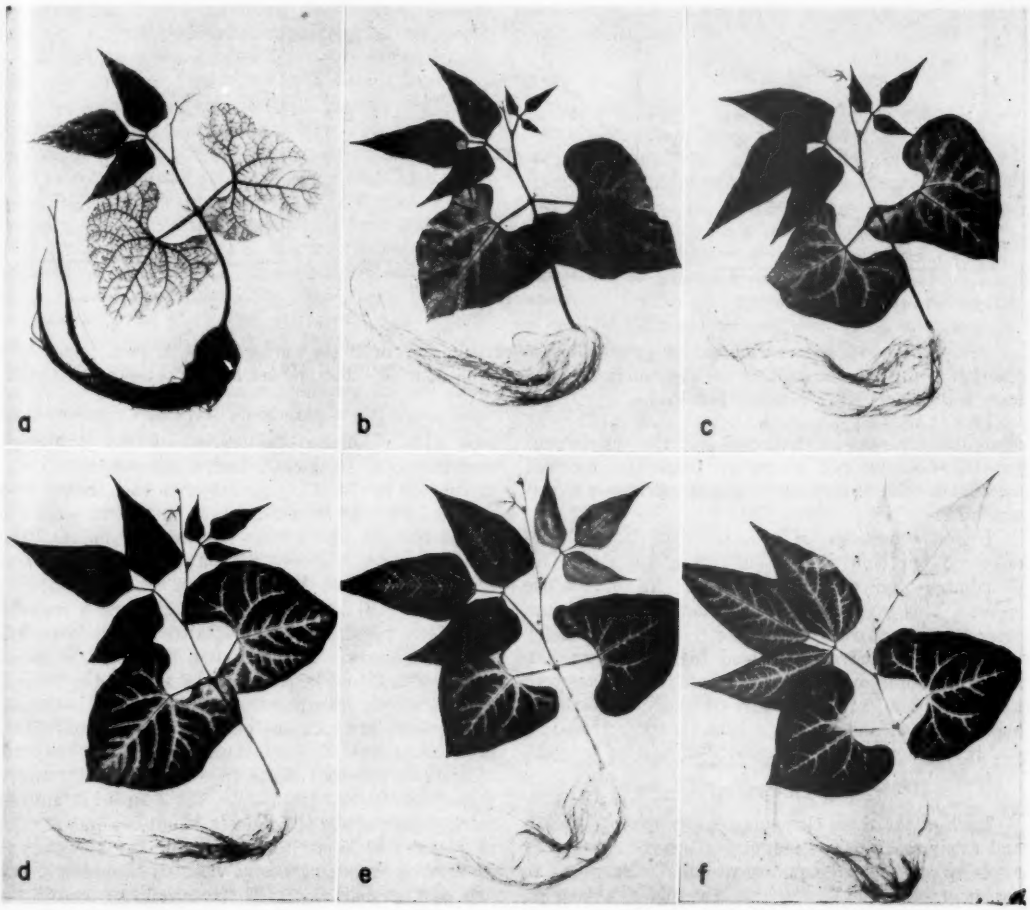

FIG. 4. The progressive distribution through a 96-hour period of a single aliquot (88 μc/L/plant) of Ca-45 administered via the nutrient solution during a 1-hour period. Exposure of dried plant to film = 60 days. The time sequence, in hours, was: a = 0, b = 6, c = 12, d = 24, e = 48, and f = 96.

val, possessed a portion of the mobile fraction, and conversely, had there been no circulation the newly formed leaves and roots would have been devoid of it.

The roots retained a rather uniform labeled sulfur concentration, both in the root tissue initially immersed in the labeled sulfur solution and in the new root growth made subsequent to the S-35 treatment. This turnover in older tissue and deposition in younger rapidly growing tissue is again the mark of mobility. The intense points of S-35 radioactivity upon the roots appeared to be due to colonies of sulfur accumulating microorganisms present upon them.

The calcium distribution pattern indicated marked immobility of this element in the phloem of the bean plant. The initial delivery in the transpiration stream was not followed by a subsequent removal, even from mature tissues, so that those tissues which were elaborated following the initial delivery contained no Ca-45, or very little of it. Likewise, the root apexes became progressively deficient in Ca-45 as they were extended by growth during the 96-hour migration period. These findings are consistent with the view that calcium is normally immobile in the phloem.

The relatively slow delivery of Ca-45 to the aerial parts of the plant is of interest. At the close of the 1-hour absorption period, the concentration of the tracer within the roots is very high, but it has largely moved upward by the close of the 6-hour migration period. The plant representing the 6-hour migration period (fig 4 b) then illustrates the pattern of initial delivery of this element better than the plant taken at the close of the 1-hour absorption period. There is little difference in the distribution pattern thereafter, for those tissues which are subsequently formed by growth contain only the original supply much

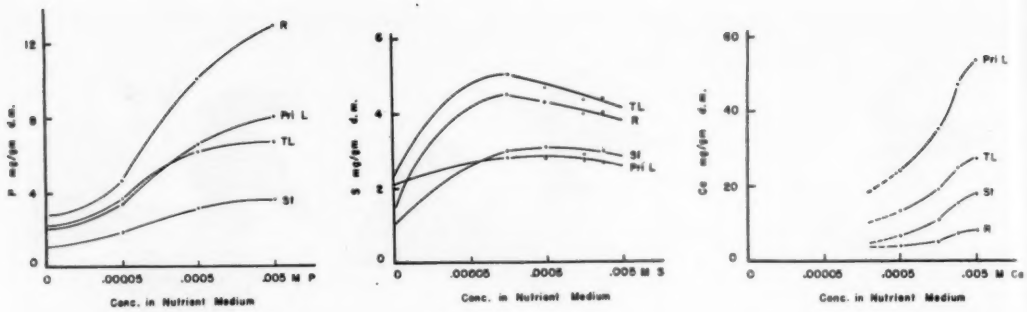

FIG. 5. The normal distribution of phosphorus, sulfur and calcium in the various parts of bean plants as a function of the concentration of the element in the nutrient solution. R = roots, St = stem plus petioles, Pri L = primary leaf blades, T.L. = trifoliate leaf blades.

diluted. All calcium required for the subsequent growth was obtained currently from the nutrient medium as the previously acquired calcium was unavailable.

Under conditions of non-mobility, the calcium delivered by the transpiration stream cannot be redistributed and continues to build up in mature leaves. This is manifest by the normal high calcium concentration of the oldest leaves, i.e., the primary leaves, and for leaves in general (fig 5). The radioautograms of figure 4 stand as conclusive proof of the almost complete immobility of calcium after being once deposited and furnish a base, or control, against which the mobility of S-35 and P-32 can be judged.

DISCUSSION

Each of the three elements i.e., phosphorus, sulfur, and calcium, displayed unique features of circulatory capacity and served to illustrate the wide range in the degree of mobility within the plant. Calcium, being largely immobile, supplied the basis upon which the peculiarities of the two mobile elements were contrasted. The period during which sulfur remained mobile was limited, presumably by the tenacity of its capture in metabolic reactions within young leaves. Phosphorus remained mobile either because of the transient nature of its capture reactions or failure of active tissues to remove the mobile fraction quickly.

From the data of this and earlier studies (18) it is evident that during the course of the growth of each succeeding trifoliate leaf there is, in addition to direct delivery, an importation of phosphorus from the more mature leaves. Each leaf then passes through a stage where it imports only to a stage where it may participate in an active exchange of the phosphorus supplied via the transpiration stream. The movement of phosphorus from the leaf occurs in the phloem (7) and the export is toward the root or the stem tip depending on the position of the leaf in question. All leaves export some material to the stem apex, but they export to the root in amounts which are proportional to the proximity of the leaf to the root (18). During the course of the downward movement, a significant lateral movement to the xylem occurs (7, 4), this material then being in a position for the reascension of the stem and the completion of one circuit within the plant. The time sequence for reascension of the phosphorus reaching the root has not been worked out, but Kursanov (19) has supplied data showing a reascension, from roots, of carbon labeled metabolites originating in leaves. This occurred, in significant quantity, within 3 to 6 hours from the time of the original photosynthetic incorporation of the labeled carbon.

A significant portion of the leaf exported phosphorus has been shown to move directly up the stem through the phloem tissue (8) which is in agreement with previous reports (6, 25, 13). In addition, upward movement in the phloem is implied in the work of Mason and Maskell (20) showing the accumulated phosphorus in young cotton bolls to be vastly in excess of that delivered in the tracheal sap, and in the work of Burstrom (12) showing phosphorus movement into the buds of Carpinus and Fagus to be in excess of the quantity which the xylem sap was capable of delivering at its current concentration. Upward movement in the phloem, at least near the stem tip, has been considered possible since the work of Curtis (14), and it is not necessary to postulate that xylem delivery is the exclusive means of transport to stem apexes.

A case for the sustained circulation of phosphorus seems very good despite the complicating effects of interpretation resulting from the high retention of tracer phosphorus by the root. All studies pertaining to this topic support the same conclusion.

There are certain points relative to the upward delivery of mineral nutrients in the transpiration stream which are best discussed in connection with the S-35 tracer studies. It has been shown that the labeled sulfur from the root moved directly into the younger leaves in relatively greater quantity than into the older primary leaves. The by-passing of the older leaves for the younger ones is quite evident. The mechanics of the preferential delivery might be con-

sidered to be based on three phenomena. First, there is an indication that transpiration per unit area of leaf surface is greater from very young leaves than from mature ones (21) so xylem delivery to young leaves may be accentuated. Second, there exists the possibility of a loss of sulfur from the transpiration stream to the phloem traces leading to the young leaves which are still importing via these traces. Third, mature leaves may export via the phloem a portion of their xylem delivered sulfur directly to the young leaves and stem apexes, as mentioned above. Of course, these mechanisms could be operative for other substances as well, i.e., other minerals, metabolites, etc. The evidence for a preferential delivery of sulfur to young growing leaves, over mature ones, in the direct delivery of sulfur from the root is far better than the explanation for it. The present study does not allow a discrimination between the suggested possibilities.

A very high degree of immobility was displayed by calcium. The initial step in its delivery by the transpiration stream constituted the final step in its distribution; it did not re-circulate. This is consistent with the view that calcium is normally immobile in the phloem (9, 3, 27, 10 and others). For this reason it is apparent that a continuous supply of calcium, rather than an intermittent one, is essential for the normal growth of plants.

Recently, in the work of Ferrell (15), and Bukovac et al (11), there is evidence that under certain circumstances calcium may actually be redistributed. In the former case the buds of white pine trees, formed during two consecutive spring seasons following application of Ca-45 to the plants, were unusually high in the tracer. In the latter case a migration of Ca-45 occurred from bean leaves which had been anesthetized with ether. In neither case is it mandatory to assume that redistribution took place via the phloem. In fact evidence of calcium immobility in normal phloem is now so strong that exceptions would be interesting.

The direct movement of root-absorbed sulfur into the meristematic tissues of the stem tip was more rapid than for phosphorus or calcium. One might conclude that the mechanism of sulfur transport across the cortex is less complicated than that of phosphorus or calcium. This may be related to the fact that both phosphorus and calcium were retained by the root in relatively high concentration, i.e., the P-32 for the whole of the 96-hour period and the Ca-45 for the first few (not over 6) hours. After its temporary retention the Ca-45 ascended the stem in the transpiration stream. One would judge that if the three were simultaneously applied the sequence of appearance of tracers in the apical meristems would be S, P, Ca, and the reason for the difference should be sought in the absorption mechanisms. Rothstein and Hayes (26) have shown an interesting time sequence in the absorption of K, P, and Mg or Mn so that differential rates of absorption and movement across the whole cortex is perhaps not an impossible concept.

Summary

The circulation patterns for P-32, S-35 and Ca-45 in the bean plant were determined, by radioautographic means, from single aliquots of tracer administered to the roots during a one-hour period. A portion of the radioactive isotope of phosphorus displayed sustained circulation throughout the plant during the whole of the 96-hour experimental period. The S-35 was originally mobile but circulation was rapidly curtailed by metabolic capture in young leaves. The Ca-45 did not recirculate following its initial delivery via the transpiration stream.

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RELATION OF POTASSIUM NUTRITION TO THE TRANSLOCATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN TOMATO PLANTS[1,2]

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In tomato plants the level of phosphorus at the time of application of 2,4-dichlorophenoxyacetic acid (2,4-D) has been shown to have a marked effect on the translocation of this plant growth regulator (8). Scott (9) found that application of potassium to potassium-deficient morning glory and zinnia plants three days before treatment with 2,4-D resulted in a significant decrease in elongation of the main axis as compared with similarly treated plants given potassium at the time of application of 2,4-D. Moreover, he found a significant increase in length of axillary branches on the plants that had received the potassium for the three additional days. These indirect criteria suggested that the potassium level at the time of 2,4-D application affected translocation of the growth regulator. It was decided, therefore, to study the effect of potassium nutrition on the translocation of 2,4-D using similar and also more direct criteria.

Experimentation

Tomato seeds (var. Stone) were planted in quartz sand in 4-inch glazed pots and watered with a low potassium nutrient solution for 29 days. The seedlings were then thinned to two uniform plants per pot and were watered with a nutrient solution containing no potassium for 11 days at which time they exhibited pronounced deficiency symptoms. Four series of 102 plants (51 pots) each were subdivided into two groups, one of which was to be treated with 2,4-D and the other not so treated. The plants in series A were watered with a complete nutrient solution (plus potassium) three days before applying 2,4-D to the treated group; those in series B were similarly given potassium 8 hours before applying 2,4-D; those in series C received potassium at the time of application of 2,4-D; those in series D were grown without potassium until 24 hours after application of 2,4-D before being watered with the complete solution. Ten plants in each series were treated on the terminal leaflet of the 3rd true leaf with 0.025 ml of 60 % ethanol containing 0.5 % Carbowax 1500 and 50 μg of 2,4-D labeled in the carboxyl position with C^{14}. Forty-one additional plants in each series were treated similarly with unlabeled 2,4-D. Stem curvatures were measured 18 hours after application of the 2,4-D.

Twenty-four hours after applying the 2,4-D the 10 plants in each series which had been treated with 2,4-D-1-C^{14} were harvested. The roots were washed, and the plants segmented to avoid the possibility of movement of radioactive material subsequent to harvesting (8). The plants were then placed in a

[1] Received revised manuscript April 23, 1958.
[2] This work was done under Contract No. AT-(40-1)-1043 with the U. S. Atomic Energy Commission.

plant press and dried at 60° C in a vacuum oven. After drying, the plants were mounted on paper, placed against Ansco Non-Screen X-ray film in the dark, and left there for 30 days after which the films were developed.

All plants which had been treated with 2,4-D, but which were not harvested as described above had the treated leaves removed 24 hours after application of the growth regulator. A similar leaf was removed from each control plant which had received no 2,4-D. All these plants were allowed to grow for 15 days after application of the 2,4-D at which time the plants were cut off at the sand level and their individual fresh weights determined.

Several similar experiments were performed with essentially the same results.

Results and Discussion

The average stem curvature of the plants in each of the three series which were given potassium prior to application of 2,4-D was significantly greater than that of the plants which were not given potassium until 24 hours after treatment with 2,4-D (table I). Moreover, the stem curvatures resulting from treatment with 2,4-D were significantly greater when the plants received potassium three days before treatment than when they received it 8 hours before or at the time of treatment. There was also a significant inhibition in growth subsequent to application of 2,4-D in those plants which obtained potassium prior to or at the time of application of the growth regulator (table I). These results demonstrate quite definitely that the effects of applied 2,4-D on potassium deficient tomato plants are enhanced by giving the plants potassium prior to application of the growth regulator.

As previously indicated (8), we believe that both stem curvature and inhibition of the growth of tops are fairly reliable indices for the measurement of translocation of 2,4-D in tomato plants. Using these criteria, it appears that the translocation of 2,4-D in tomato plants is markedly affected by the potassium level.

The radioautograms (fig 1) indicated that a considerable amount of radioactive material moved through the stems, hypocotyls, and even into the roots of most of the plants which were given potassium three days before the labeled 2,4-D was applied to them. The movement of radioactive material was considerably greater in those plants which received potassium 8 hours before application of the 2,4-D-1-C^{14} than in those which received the potassium later but was less than in those plants which received the potassium three days before. Six of the plants which received potassium 8 hours before application of the 2,4-D had radioactive material in the roots, whereas no plants which received potassium later exhibited this condition. There was a greater amount of radioactivity in the upper parts of the stems and hypocotyls of those plants which were given potassium and 2,4-D at the same time than in those plants which were given potassium 24 hours after application of the growth regulator. It is probable that some of the radioactivity in all the plants described above was in compounds other than 2,4-D (1, 2, 15), but the curvature responses and inhibition in growth certainly indicate that some of the C^{14} remained in 2,4-D or an active derivative of it.

The evidence from the radioautograms, the curvature measurements, and the weight measurements suggests quite strongly that the level of potassium in tomato plants at the time of application of 2,4-D has a pronounced effect on the translocation of the growth regulator.

Many workers (3, 4, 6, 7, 13, 14) have demonstrated that a relationship exists between the translocation of 2,4-D and the translocation of carbohydrates in plants. There is considerable evidence that

Table I

Relation of Time of Adding Potassium to the Effect of 2,4-D on Potassium Deficient Tomato Plants

Series	Time of adding K in relation to time of application of 2,4-D	Mean stem curvatures of treated plants 18 hours after treatment with 2,4-D		Mean fresh wts of tops 15 days after treatment with 2,4-D			
				Treated with 2,4-D		Controls, no 2,4-D	
		No. of plants	Degrees curvature with std error *	No. of plants	Wts (g) with std error **	No. of plants	Wts (g) with std error †
A	3 days before	51	63 ± 4.5	38	0.84 ± 0.15	51	2.40 ± 0.18
B	8 hrs before	50	49 ± 3.8	38	0.52 ± 0.13	50	1.85 ± 0.13
C	0 hrs before	51	51 ± 3.3	38	0.77 ± 0.14	51	1.50 ± 0.09
D	24 hrs after	50	30 ± 2.3	37	1.58 ± 0.17	51	1.23 ± 0.08

* Mean curvatures in Series A, B, and C are significantly greater than that of Series D at the 0.01 level. Mean curvature in Series A is significantly greater than that of Series B or C at the 0.05 level.

** Mean weight of treated plants in Series D is significantly greater than that of the treated plants in Series A, B, or C at the 0.01 level.

† Mean weight of treated plants is significantly less than that of control plants in Series A, B, or C at the 0.01 level.

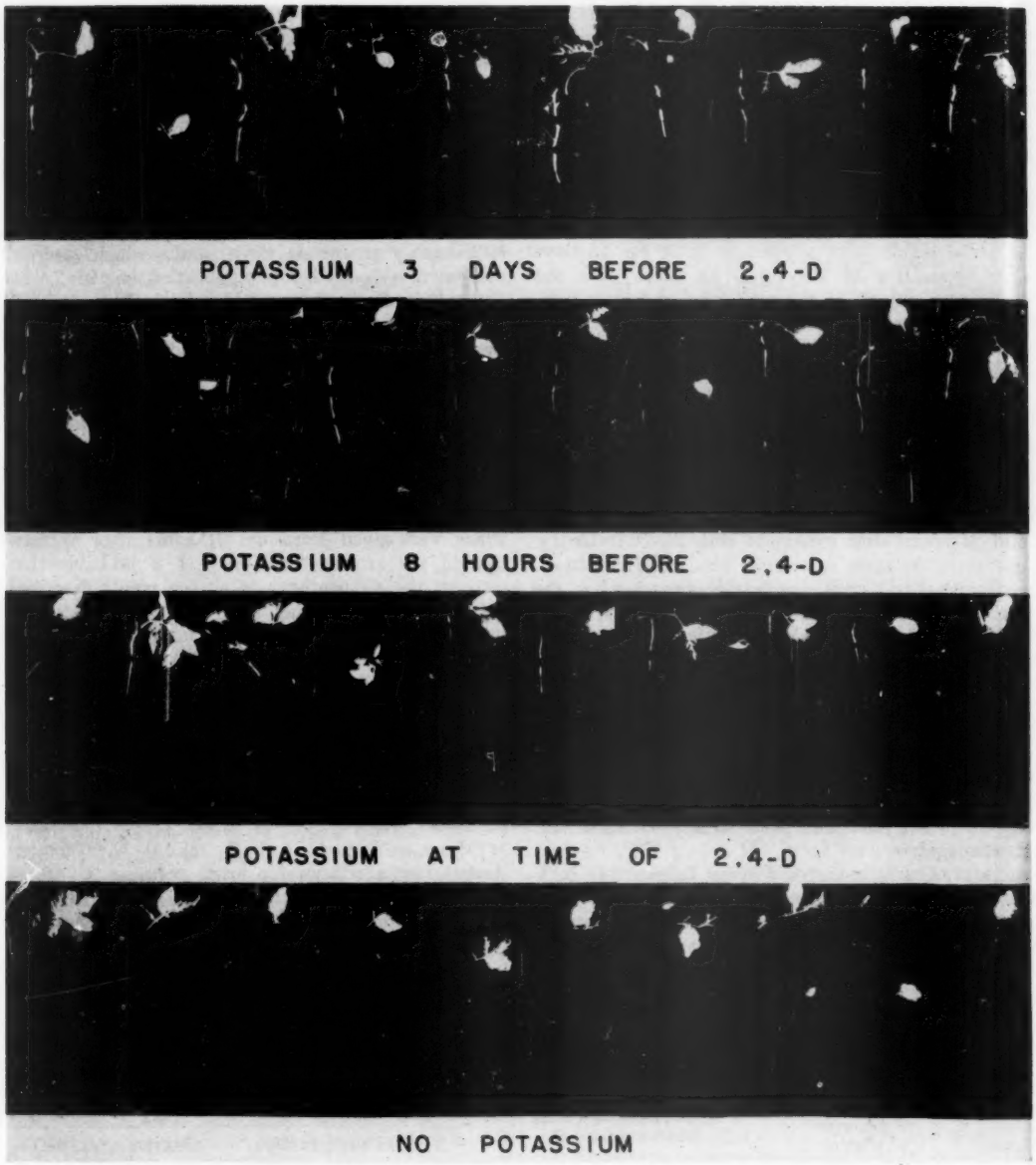

FIG. 1. Prints of radioautograms of K-deficient tomato plants which were given K at different time intervals before being treated with C^{14}-labeled 2,4-D.

potassium plays an important role in CO_2 assimilation and carbohydrate accumulation in many plants, including tomatoes (5, 10, 11, 12). It is quite possible, therefore, that the decrease in rate of translocation of 2,4-D through the tomato plants with a decrease in potassium level was due to a lowering of the carbohydrate supply. If this is true, it is probable that potassium and phosphorus affect translocation of 2,4-D by different means (8).

SUMMARY

Potassium deficient tomato plants which were given potassium before or at the same time radioactive 2,4-D was applied had significantly greater stem curvatures, greater inhibition in growth, and greater movement of radioactive material than did plants which were given potassium 24 hours after application of the growth regulator. Those plants

which received potassium three days before applying the 2,4-D showed the greatest stem curvatures and movement of radioactive material into the lower parts of the hypocotyls and roots.

The evidence suggests that the translocation of 2,4-D in potassium deficient tomato plants is markedly affected by the level of potassium in the plants.

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Chloroplast Pigments and Chromatographic Analysis—Harold H. Strain. Phi Lambda Upsilon, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania. 180 pages. Paper cover. 1958. $2.50.

This book represents the Thirty-second Annual Priestley Lectures. The first three of these five lectures are concerned with the isolation and chromatography of chloroplast pigments from a variety of plants. The fourth is a general commentary on the interrelations of solute, solvent and sorbent, and the fifth deals in a similar manner with electrochromatography (primarily paper electrophoresis). A series of appendices, arranged taxonomically, relate the general appearance of the chloroplast chromatograms, especially the presence or absence of α-carotene. These appendices comprise almost one fourth of the book.

Although in typescript, the text is very readable and the figures are quite clear.

Lecture I is essentially historical, concluding with the now well-known experience that no one system can separate all the pigments adequately. This anguish has been voiced repeatedly for amico acids, steroids, etc.

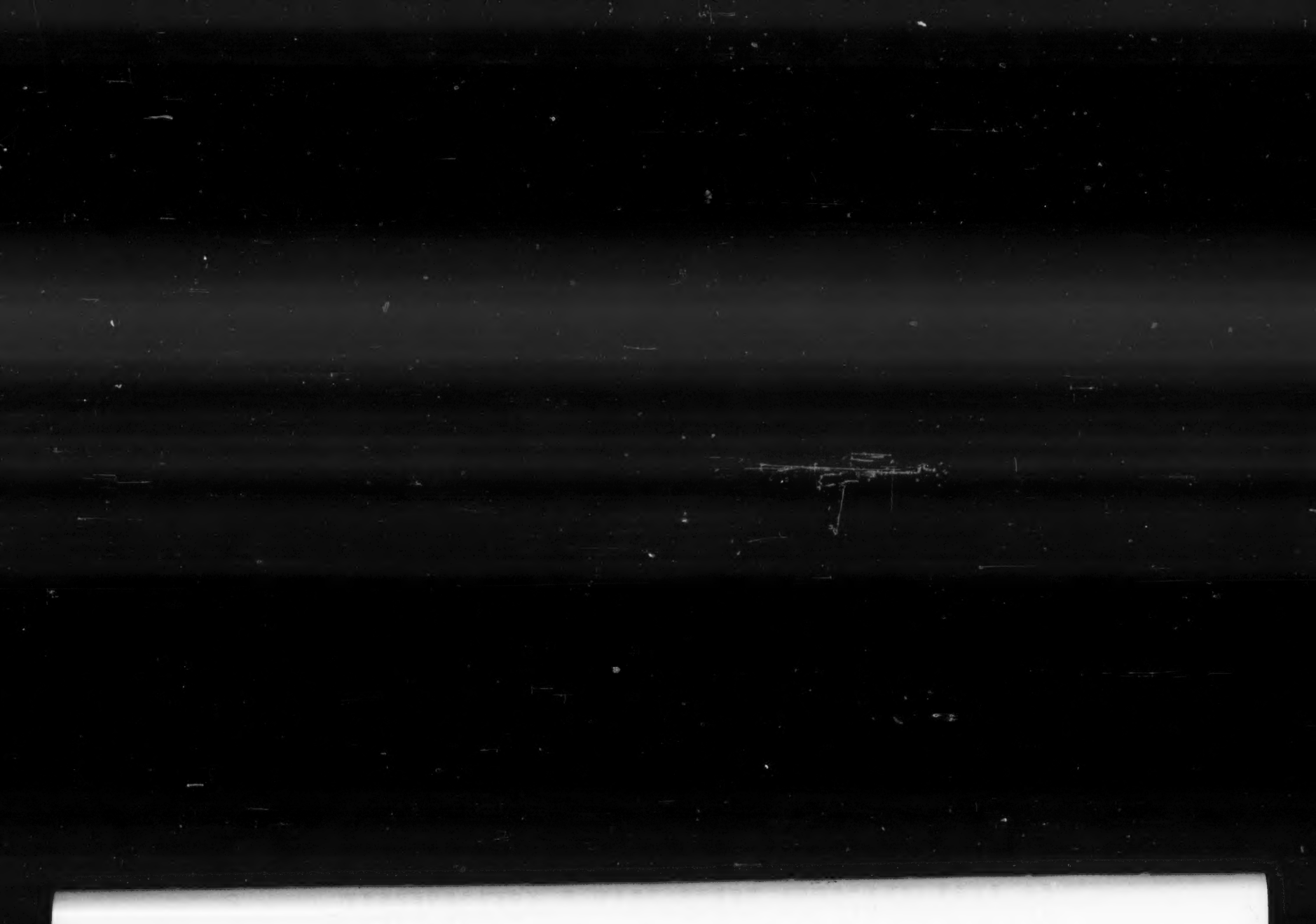
Lecture II relates the concern of the author for changes in the pigments attending extraction and chromatography; provides the author's general methodology and discusses the distribution of chloroplast pigments taxonomically, primarily among the higher green plants and algae.

Lecture III includes a description of the chloroplast pigments in Euglena, Heterokontae, diatoms (Bacillariophyceae), dinoflagellates, red, brown and blue-green algae (except for the bilins) and the various isomerization products of chlorophylls a, b and d and the chlorophyllides. There is also a short discussion of the chromatography of allomerization products, as well as further brief discussion of the taxonomic aspects of pigment distribution.

Lecture IV concerns the interrelations of solute, solvent and absorbent, stating the roles of the latter, and the relation of molecular structure to sorption, especially as related to sorption sequence of carotenoid pigments.

The final lecture delineates in a general manner the various factors involved in electrochromatography. Several examples are provided of inorganic separations using different principles, including two-way electrochromatography and chromatography + transverse electrochromatography.

The text covers the endeavors of many years and is a personal one. Consequently data on bactochlorophyll (bacteriochlorophyll), Chlorobium chlorophyll, phycocyanin and phycoerythrin are conspicuous by their absence. It does not intend to probe minutely into any aspect, but ample references are provided for those who wish to pursue the studies further. Except for some data in Lectures II and III, and the Appendices, the plant physiologist of average experience may find little that is novel. Yet to the beginner, and certainly to the taxonomist, the text may be rather useful.—S. Aronoff, *Depts. of Botany and Chemistry, and the Institute for Atomic Research, Iowa State College.* Written during the tenure of a National Science Foundation Senior Postdoctoral Fellowship at the National Institutes of Health, Bethesda, Md.

Topics in Microbial Chemistry—F. M. Strong. John Wiley and Sons, Inc., New York. 166 pages. 1958. $5.00.

This little book contains the subject matter of a short series of lectures presented at the Institute of Microbiology, Rutgers. It describes the part played by investigators at the University of Wisconsin in elucidating the chemical nature and, to a lesser extent, the physiological function of antimycin A, coenzyme A and kinetin. The chapters on antimycin A and kinetin, in particular, provide a valuable insight into the problems confronting those who would isolate and identify complex biological substances. At the same time the ingenious techniques by which many of these problems have been solved are presented in enough detail to be intelligible. As monographs the three chapters are certainly incomplete; the necessary brevity and the personal approach of the author have made this inevitable. However, any lack of coverage is more than compensated for by the first-hand knowledge of the work which the author obviously possesses. As a dissertation on the practical problems of biochemical research this account by one who was there has pedagogic merits rarely found in the works of the historians of science who usually write textbooks.—Norman Good, *Botany Department, University of Minnesota, Minneapolis.* For 1957-1958, on leave from Science Service, Canada Department of Agriculture, London, Ontario.